CHEMICAL REVIEWS

REVIEW

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# Free Radical Lipid Peroxidation: Mechanisms and Analysis

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## 1. INTRODUCTION — MECHANISM OF AUTOXIDATION (PEROXIDATION)

Oxygen is indispensable to metabolism and energy production for almost all life forms. However, reactive oxygen species (ROS) generated from life processes can cause damage to cells, tissues, and organs. In response to this threat from oxygen, organisms utilize multiple layers of antioxidant defense, damage removal, and repair or replacement systems to enjoy life in an oxygen-rich environment.[1] Oxidative stress, the disturbance of pro-oxidant and antioxidant systems in favor of the former, is a topic of a substantial research effort involving studies of the basic oxidation reaction mechanisms at the fundamental level and the clinical management of oxidative assault at another.

Lipids are essential components of cell membranes that maintain structure and control the function of cells. They are primary targets of the attack by ROS such as oxygen free radicals, and the oxidation of lipid is associated with various pathological states.[2] Lipid peroxidation, or reaction of lipid with molecular oxygen, has been an intensive research area for decades,[3] and in recent years, the complex nature of the lipid peroxidation process and its potential biological significance have been attracting scientists from across many disciplinary fields, ranging from chemistry and biochemistry to biology and clinical science. Lipid peroxidation has been implicated in several human diseases and exposures such as atherosclerosis,[4] cancer,[5,6] diabetes,[7] chronic alcohol exposure,[8] acute lung injury,[9,10] as well as the neurodegenerative disorders[11] that include Alzheimer's[12] and Parkinson's disease.[13] Efforts have been devoted to understanding the mechanism of lipid peroxidation and preventing the deleterious effects of this process.

It is the aim of this Review to discuss the chemistry associated with each of the steps of the free radical chain sequence in the reaction of lipids and molecular oxygen. The primary reactions of lipid peroxidation include hydrogen-atom abstraction by peroxyl or alkoxyl radicals; oxygen addition to carbon radicals; peroxyl radical fragmentation or rearrangement; peroxyl radical

**Special Issue:** Lipid Biochemistry, Metabolism, and Signaling

**Received:** March 18, 2011
**Published:** August 23, 2011

**Scheme 1. Reactions in the Free Radical Chain Oxidation Mechanism**

**Initiation:**

$$In{-}In \longrightarrow In^{\bullet} + In^{\bullet} \quad (1)$$

$$In^{\bullet} + L{-}H \xrightarrow{k_{iLH}} In{-}H + L^{\bullet} \quad (2)$$

**Propagation:**

$$L^{\bullet} + O_2 \xrightarrow{k_{perox}} L{-}OO^{\bullet} \quad (3)$$

$$L{-}OO^{\bullet} + L{-}H \xrightarrow{k_p} L{-}OOH + L^{\bullet} \quad (4)$$

**Termination:**

$$2\ L{-}OO^{\bullet} \xrightarrow{k_t} [L{-}OO{-}OO{-}L] \quad (5)$$

$$[L{-}OO{-}OO{-}L] \longrightarrow \text{Non-radical products (NRP)} + O_2 \quad (6)$$

$k_{iLH} = 6 \times 10^1\ M^{-1}s^{-1}$ $k_p = 6 \times 10^1\ M^{-1}s^{-1}$ for linoleate In, Initiator; L, Lipid

$k_{perox} = 10^9\ M^{-1}s^{-1}$ $k_t = 1 \times 10^5$ to $10^7\ M^{-1}s^{-1}$

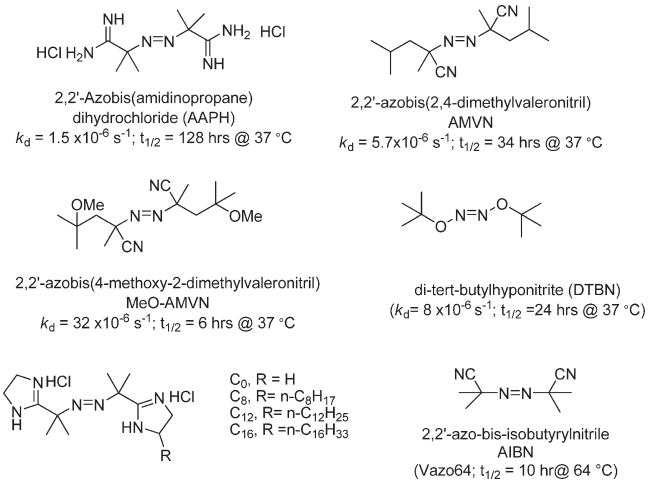



**Figure 1.** Selected azo initiators used in lipid peroxidation studies.

addition to carbon−carbon double bonds or cyclization; and peroxyl−peroxyl termination.[14] There are a number of reviews of lipid peroxidation that have appeared in recent years, particularly related to the biology of lipid oxidation products.[15−19] We focus here principally on the basic free radical chemistry of lipid peroxidation and recent developments for the detection and analysis of free radical lipid peroxidation products and their biological relevance. The analysis of lipid peroxidation products has been particularly important in the advancement of research in this field because of the complexity of product mixtures.

The free radical chain oxidation of organic compounds has historically been referred to by organic and physical chemists as autoxidation.[3,20−29] The peroxide products of the reaction can serve as initiators of the process under certain conditions, and under those circumstances, the rate of oxygen consumption increases over time as peroxide products are formed. The mechanism of free radical autoxidation can be understood by a chain process consisting of initiation, propagation, and termination steps as shown in Scheme 1. When the compound undergoing reaction is a lipid or other compound of biological importance (protein, carbohydrate, nucleic acid), the process is normally referred to as peroxidation. Cellular membranes contain significant amounts of polyunsaturated fatty acids that are esterified on phospholipids, as well as free cholesterol. These lipids are the primary targets for free radical attack, and thus free radical lipid peroxidation has a profound impact on the biology of living systems.

### 1.1. Initiation

In the initiation step, the key event is the formation of a lipid radical L•. In a biological system, cellular membrane lipid oxidation can be induced by exogenous physical and chemical reagents, such as air pollution, smoking, UV-light, or ionization radiation. On the other hand, multiple endogenous enzymatic systems can also generate free radicals. These enzymes include NADPH oxidase, xanthine oxidase, uncoupled nitric oxide synthase, and cytochrome P450. Free radicals can also be produced from the electron transport chain (ETC) in mitochondria. It is estimated that 1−5% of the electrons flowing through the ETC can leak and react with oxygen to produce superoxide.[19] In vitro oxidation of unsaturated fatty acid-containing lipids can be initiated by a variety of methods, including the use of transition metals such as copper and iron, enzymes, hydroxyl radical, gamma irradiation, and cultured cells that can produce reactive oxygen species or nitrogen species. Several enzymes have been employed to mimic in vivo radical sources, such as 15-lipoxygenase and myeloperoxidase.[30−32] Protein tyrosyl radicals generated during the normal turnover of prostaglandin $H_2$ synthases (PGHS) can also initiate lipid peroxidation. In the PGHS-peroxidase site, reduction of a hydroperoxide yields a ferryloxo protoporphyrin radical cation, and through intramolecular electron transfer, the radical generates a tyrosyl radical in the PGHS-cyclooxygenase site that catalyzes oxygenation of arachidonic acid in its normal function. Yet arachidonyl radicals apparently escape from the enzyme active site on occasion, and this results in the initiation of a radical chain reaction.[33] Tyrosyl radicals in myoglobin and hemoglobin also initiate peroxidation reactions that play a potentially significant role in rhabdomyolysis, subarachnoid hemorrhage, malaria, and sickle cell disease.[34] Singlet oxygen and peroxynitrite have also been used to generate peroxide products, but the singlet oxygen reaction does not proceed by a radical mechanism, and mechanisms involving peroxynitrite are more complex than simple free radical chain chemistry.[35]

In mechanistic chemical studies, it is important to have methods that can be used for the generation of free radicals at a well-defined rate, and azo free-radical initiators have been used to fill this role. Azo compounds decompose by first-order kinetics under most conditions used to study oxidation reactions, and rate constants for decomposition have been reported under a variety of conditions. Hydrophilic, hydrophobic, and amphiphilic azo initiators have been prepared and studied.[36] Some of the azo initiators that have been used in the autoxidation of lipids and lipoproteins are shown in Figure 1.

Thermal decomposition of these azo-initiators results in the generation of molecular nitrogen and a pair of radicals. The rate constant for decomposition, $k_d$, of the azo initiator at a given temperature varies depending on the structure. For example, AAPH decomposes at 37 °C in methanol with $k_d = 1.5 \times 10^{-6}\ s^{-1}$, $\tau_{1/2}$ = 128 h (half-life); and AMVN decomposition at the same temperature in benzene gives a value of $k_d = 5.7 \times 10^{-6}\ s^{-1}$, $\tau_{1/2}$ = 34 h, while MeOAMVN has $k_d = 32 \times 10^{-6}\ s^{-1}$, $\tau_{1/2}$ = 6 h.[37,38] In the case of AAPH, AMVN, and MeOAMVN, the two radicals produced in the bond homolysis are carbon-centered alkyl radicals. These carbon radicals readily


dx.doi.org/10.1021/cr200084z |Chem. Rev. 2011, 111, 5944–5972

add oxygen to generate two peroxyl radicals at the diffusion-controlled rate of $10^9$ $M^{-1}$ $s^{-1}$ (eq 3 in Scheme 1). On the other hand, DTBN generates two alkoxyl radicals that can initiate free radical reactions. The alkoxyl radicals generated from DTBN decomposition ($k_d \approx 8 \times 10^{-6}$ $s^{-1}$, $\tau_{1/2} \approx 24$ h at 37 °C in iso-octane) react more rapidly with LH than do peroxyl radicals (rate constant for reaction with LH, $k_{LH}$; for RO•, $9 \times 10^6$ $M^{-1}$ $s^{-1}$ and for ROO•, $6 \times 10^1$ $M^{-1}$ $s^{-1}$ when LH is linoleate). The selectivity of alkoxyl radicals is much less than that of peroxyl radicals.[39−41]

## 1.2. Propagation

### 1.2.1. Oxygen Addition.

$$\underset{\text{lipid radical}}{L\bullet} \overset{O_2}{\rightleftarrows} \underset{-O_2\ \text{peroxyl radical}}{L{-}O{-}O\bullet}$$

In one of the propagation steps of peroxidation, molecular oxygen is added to the carbon-centered radical L• to generate a peroxyl radical (LOO•) (Scheme 1, eq 3), and it is the peroxyl radical that is the principal chain-carrying species under most circumstances. Oxygen addition to carbon-centered radicals occurs at or near the diffusion-controlled rate at oxygen pressures above 100 mmHg.[42] It is noteworthy that oxygen concentration varies in different tissue/organ and under different pathophysiological conditions; thus the rate of oxygen addition to a carbon-centered radical falls with the decreasing of oxygen tension, vide infra.[27] Subsequent reactions of peroxyl radicals tend to be relatively slow on the time scale of other radical reactions, so it is the peroxyl species that is the dominant radical present in the chain.

Polyunsaturated fatty acids and esters are particularly prone to undergo autoxidation, linoleate and arachidonate being examples of fatty acid esters that are oxidizable (Figure 2). The C−H bonds at the bisallylic positions, C-11 in linoleate and C-7, C-10, and C-13 in arachidonate,[43] are the weakest bonds in the molecules, and the hydrogen atoms at these positions are preferentially abstracted by a peroxyl radical. The bond dissociation enthalpy (BDE) at these bisallylic positions is about 78−80 kcal/mol.[44] Oleate, a monounsaturated fatty acid, is much less prone to undergo oxidation than are the homoconjugated lipids like linoleate and arachidonate. The allylic C−H bonds at C-8 and C-11 of oleate are about 10 kcal/mol higher in energy than the bis-allylic substructure in linoleate, and oleate free radical oxidation occurs less readily than the process for the polyene substrates.[45] For cholesterol, the allylic C−H bond at C-7 is subject to abstraction by peroxyl radicals, and for 7-dehydrocholesterol (7-DHC), the C-9 and C-14 hydrogen atoms are vulnerable to attack.[26,46]

The important primary carbon radical intermediates in lipid peroxidation have the general structures shown in Figure 3. The carbon radicals formed from the polyunsaturated fatty acid esters and from 7-DHC are highly delocalized, while those formed from cholesterol or oleate are more localized, spanning only three carbon centers. The carbon radical formed from linoleate is delocalized over the five carbons from C-9 to C-13, and analogous radicals derived from arachidonate, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are also highly delocalized.[47−53] Three such radicals are formed from arachidonate, four from EPA and five from DHA according to the number of reactive centers on each of these fatty acids. Cholesterol gives one delocalized allyl radical that results from abstraction of one hydrogen atom at C-7, and 7-DHC gives rise to two highly

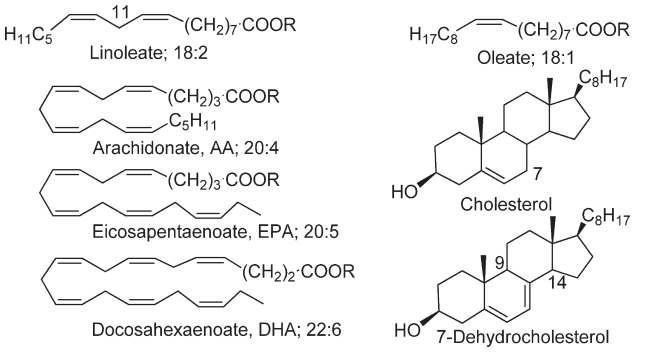



**Figure 2.** Highly oxidizable lipid substrates.

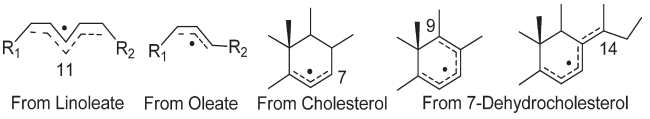



**Figure 3.** Delocalized carbon chain carrying carbon radicals.

delocalized radicals after abstraction of hydrogen atoms at either C-9 or C-14 of the sterol.

*1.2.1.1. Kinetic Products of Oxygen Addition.* The pentadienyl carbon radicals derived from linoleate and the other fatty acid esters have the odd electron spin distributed principally on the two terminal carbons and one central carbon of the five-carbon framework. Oxygen addition could, in principle, occur at these three positions, but it has only been recently that products resulting from addition of oxygen to the central carbon of the radical have been isolated and identified.[54−57] The C-11 addition product from linoleate is only observed under conditions where an exceptionally good hydrogen atom donor is present in the medium. Oxidation of methyl linoleate in the presence of 1 M α-tocopherol, for example, gives rise to a kinetic product mixture of hydroperoxides with the nonconjugated C11 hydroperoxide being the major product, Figure 4. Under these conditions of oxidation, the only other hydroperoxides isolated are the two conjugated diene products having the *Z*,*E*-geometry at the diene center. For purposes of discussion, we designate these products as kinetic products of oxidation because they result from trapping the initially formed peroxyl radical by H-atom transfer from an excellent hydrogen atom source like α-tocopherol. If α-tocopherol or other good H-atom donors are not present during lipid peroxidation, then thermodynamic products resulting from unimolecular peroxyl radical reactions that compete with H-atom transfer are observed (vide infra).[56]

Oxidation of oleate gives rise to four allylic hydroperoxides under kinetic conditions in which the initially formed peroxyl radicals abstract hydrogen atoms. Thus, abstraction at the allylic C-8 position leads to the (*Z*)-8-hydroperoxy-9-enoic and (*E*)-10-hydroperoxy-8-enoic products. Abstraction of the allylic C-11 hydrogen leads to a comparable pair of products with the hydroperoxide substituted at the C-9 and C-11 centers on the chain, Figure 4.[45]

Recent studies have led to the notion that both electronic and steric effects control the site of addition of oxygen to pentadienyl radical species. The C-9, C-11, and C-13 positions of the linoleate carbon radical are the centers where the odd-electron spin is localized, and the electronic distribution therefore makes these

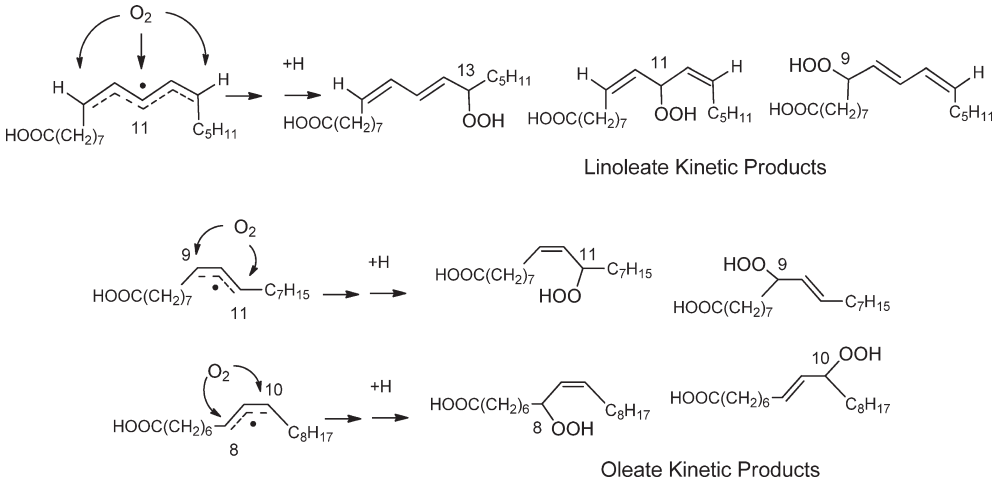



**Figure 4.** Kinetic products of oxygen addition to oleate and linoleate carbon radicals.

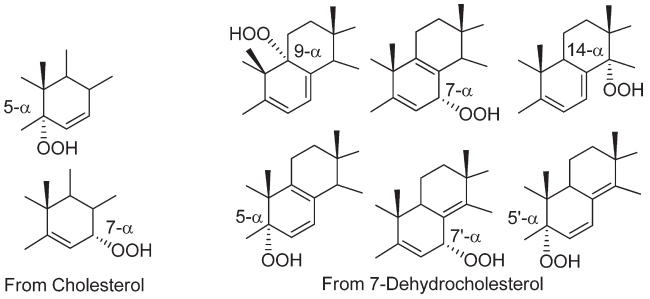



**Figure 5.** Kinetic products from cholesterol and 7-dehydrocholesterol peroxidation.

sites prone to attack by oxygen, a molecule that has two unpaired electrons. The addition of oxygen to the radical is essentially a "pairing" of the odd electron on carbon with one of the unpaired electrons on oxygen. Steric effects are also important in the reaction of oxygen with carbon radicals. Substitution of bulky groups on one end of a pentadienyl radical leads preferentially to the formation of products formed by addition of oxygen to the central position and the other end of the radical.[56] In short, oxygen adds to the most accessible site on the radical that has excess electron spin. In the case of the allyl radicals derived from oleate under kinetic conditions, the four products shown in Figure 4 are formed in about equal amounts.[45] Electron spin distribution and steric effects apparently lead to a balanced product distribution in this case.

The kinetic product distribution from cholesterol and 7-DHC has not been well-defined, but on the basis of the results of experiments with linoleate and oleate, one can anticipate that the kinetic products from cholesterol would include the 5 and 7 hydroperoxide,[58] and the 5, 7, 9, and 14 hydroperoxides[59] would form from 7-DHC, Figure 5. The $\beta$ face of these sterols is relatively protected by axial methyl groups, and the $\alpha$ face is thus the likely site of reaction.

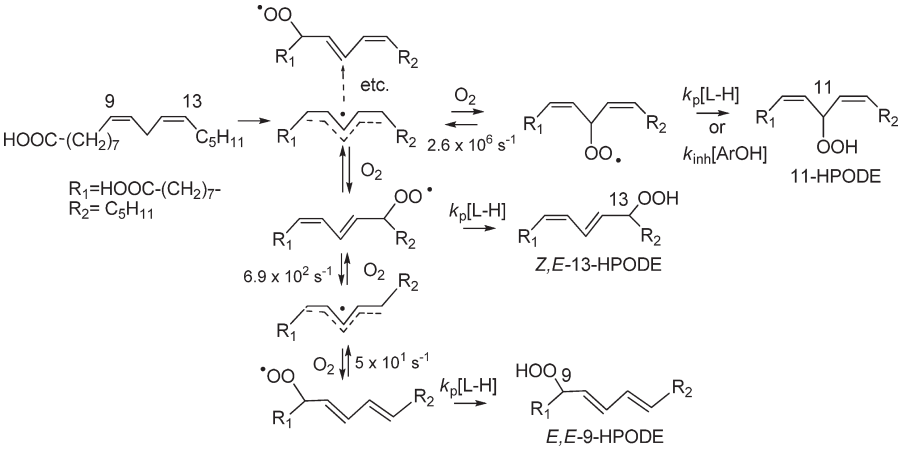
*1.2.1.2. Thermodynamic Products of Oxygen Addition.* Oxygen addition to carbon radicals is reversible,[21] and the loss of oxygen from a peroxyl radical can have a consequential impact on the distribution of products observed in a chain oxidation sequence. The consequence of reversible oxygen addition on product distribution is evident in the linoleate system and serves as an illustration of the point. Under conditions of linoleate oxidation in which good H-atom donors such as α-tocopherol are present at millimolar concentrations or higher, the three kinetic hydroperoxides are observed; see Figure 4.[57] Significant concentrations of the 11-hydroperoxide are only observed at α-tocopherol concentrations above ~0.1 M, and none of this nonconjugated product is observed in oxidation reactions carried out in the absence of antioxidant. Indeed, in bulk-phase oxidations of linoleate or for oxidations in dilute solutions, four hydroperoxides are observed, the two kinetic products having *Z,E*-diene geometry and two analogous products having *E,E*-diene stereochemistry.[50]

A partial mechanism for linoleate oxidation is presented in Scheme 2. In this scheme, oxygen addition to the first formed pentadienyl radical gives three peroxyl radicals, two of them having *Z,E*-conjugated dienes with oxygen substitution at C-9 and C-13 and one nonconjugated diene with oxygen substitution at C-11. The reverse of oxygen addition, $\beta$-fragmentation, leads to two additional peroxyl radicals having *E,E*-conjugated diene with oxygen substitution at C-9 and C-13 (for simplicity, only chemistry of the *Z,E*-9-peroxyl is shown in Scheme 2). The *Z,E*-13 peroxyl undergoes analogous reactions at similar rates.

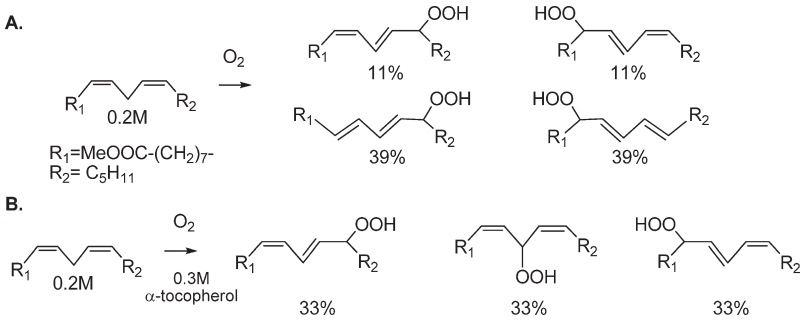
Rate constants for fragmentation of the peroxyl radicals, shown in Scheme 2, have been established by competition kinetics, and the rates depend dramatically on radical structure. Fragmentation of the nonconjugated peroxyl radical occurs with a rate constant of over $10^6$ $s^{-1}$, while the conjugated peroxyls fragment at much lower rates. A consequence of the fragmentation of peroxyl radicals as described in Scheme 2 is that the distribution of products formed from linoleate oxidation depends on the medium of oxidation. If good H-atom donors are present, then those donors trap the nonconjugated or *Z,E*-conjugated diene peroxyls giving a HPODE kinetic product set (*Z,Z*-11-HPODE, *Z,E*-13-HPODE, and *Z,E*-9-HPODE). If poor or low concentrations of hydrogen atom donors are present, then the most stable peroxyl radicals are formed, and the *E,E*-conjugated diene HPODEs are formed (*E,E*-13-HPODE and *E,E*-9-HPODE) as the major products. As an example of how H-atom donors affect product distribution, consider the oxidation of 0.2 M methyl linoleate in organic solvent at 37 °C. The product mixture formed at early stages of oxidation gives a product mixture containing no or undetectable amounts of 11-HPODE, 11%


dx.doi.org/10.1021/cr200084z |*Chem. Rev.* 2011, 111, 5944–5972

**Scheme 2. Mechanism for Linoleate Oxidation: Formation of Hydroperoxyoctadecadienoates (HPODEs) by Reversible Addition of Oxygen to Pentadienyl Radical Intermediates**



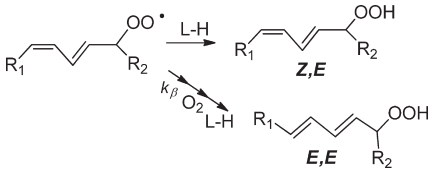

**Scheme 3. HPODE Product Dependence in the Peroxidation of Methyl Linoleate at 37 °C in Organic Solvent on Co-oxidant Availability**




each of *Z,E*-9-HPODE and *Z,E*-13-HPODE and 39% each of *E,E*-9-HPODE and *E,E*-13-HPODE, Scheme 3A. Oxidations of 0.2 M methyl linoleate under identical conditions but with 0.3 M α-tocopherol give a product mixture that contains only the two *Z,E*-conjugated diene hydroperoxides and the nonconjugated 11-HPODE in a 1:1:1 ratio of 11-HPODE to *Z,E*-9-HPODE to *Z,E*-13-HPODE, Scheme 3B.

None of the *E,E* products are formed if α-tocopherol is a co-oxidant. Thus, the linoleate product distribution provides a measure of the H-atom donating character of the medium of oxidation. If the medium is rich in good H-atom donors, then some of the nonconjugated HPODE would be expected. If the medium is poor in H-atom donors, then the *E,E*-conjugated diene products are the major products. At intermediate concentrations of H-atom donors, *Z,E* products are the major products formed. Because linoleic acid is an essential fatty acid and its esters are present at relatively high levels in the lipid pool, efforts have been made to use linoleate oxidation products, HPODEs and the corresponding hydroxy octadecadienoates (HODEs), as measures of oxidative stress in vivo. Protocols for isolation from tissues and biological fluids and analysis of the HODEs have been reported.[60−66] In assays of serum and tissue, for example, the *Z,E*- and *E,E*-HODEs have been observed and suggested as biomarkers of free radical oxidation, but no evidence for formation of the nonconjugated 11-HODE has been reported.

**Scheme 4. Competition Kinetics Determining *Z,E*/*E,E* Product Ratio**




The *Z,E* to *E,E* product ratio has also been reported in a number of studies and suggested as a means to measure the H-atom donating characteristics of the medium undergoing oxidation in vivo. The potential for use of the *Z,E*/*E,E* product ratio as a biomarker is based on the mechanism shown in Scheme 2 and expanded in Scheme 4. Formation of the *E,E* product requires β-fragmentation of the *Z,E*-conjugated diene peroxyl radical, the process indicated by a rate constant of $6.9 \times 10^2$ $s^{-1}$ in the scheme. So the critical rate competition that defines the *Z,E*/*E,E* product ratio is H-atom transfer to the *Z,E* peroxyl radical and β-fragmentation of that radical.


dx.doi.org/10.1021/cr200084z |*Chem. Rev.* 2011, 111, 5944–5972

**Table 1. Bimolecular Rate Constants for Hydrogen Atom Transfer Propagation of Lipid Substrates at 37 °C in Solution**

| H-donor | $k_p$ in benzene ($M^{-1}$ $s^{-1}$) |
|---|---|
| oleic acid | 0.9[a] |
| cholesterol | 11 ± 1[b] |
| linoleic acid | 62[a] |
| arachidonic acid | 201 ± 12[b] |
| eicosapentaenoic acid | 249 ± 14[b] |
| docosahexaenoic acid | 321 ± 32[b] |
| 8-dehydrocholesterol | 994 ± 33[c] |
| 7-dehydrocholesterol | (2.26 ± 0.04) × $10^3$[b] |
| butylated hydoxytoluene | (3.22 ± 0.32) × $10^3$[d] |

[a] 30 °C, Howard and Ingold, **1967**. [b] Xu, L; Davis, T.; Porter, N. *J. Am. Chem. Soc.* **2009**, *131*, 13037−13044. [c] Unpublished results (Xu, L; Porter, N., **2011**). [d] Inhibition rate constant.

Attempts have been made to use the *Z,E/E,E* product ratio as a means for assessing the reducing capacity of a biological medium undergoing in vivo peroxidation. The HODE *Z,E/E,E* product ratios previously reported for tissues such as liver and brain vary from 0.5 to 2.0, and plasma ratios are somewhat higher, between 2.0 and 3.0. The reported literature protocols for HODE assay in tissues involve homogenization, reduction with sodium borohydride in the presence of BHT, and ester hydrolysis with KOH to give the free HODEs. This is followed by either reverse-phase HPLC of the free acid HODEs or by conversion to TMS derivatives and GC−MS.[60−66] When sodium borohydride is replaced in the protocol by triphenylphosphine, a gentler reducing agent, HODE *Z,E/E,E* product ratios are much higher, and lower total HODE levels are found. It appears that the use of sodium borohydride in the isolation procedures leads to ex vivo oxidation reactions that are avoided if triphenylphosphine is used as the reducing agent.[67] The borohydride method that resulted in significant ex vivo oxidation gave *Z,E/E,E* HODE product ratios close to 1 for both human plasma and mouse liver. In contrast, when ex vivo oxidation was eliminated, the *Z,E/E,E* HODE product ratio determined for human plasma was about 6, and for mouse liver, the value was close to 20. It appears that the dominant HODEs formed in vivo have *Z,E* diene geometry.

### 1.2.2. Hydrogen Atom Transfer.

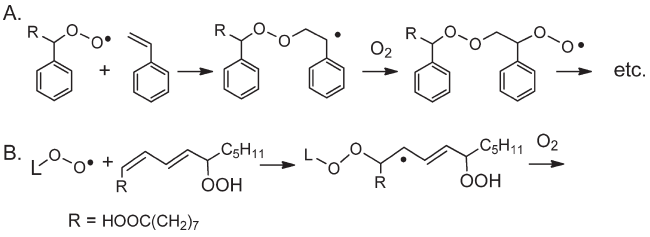
$$\mathrm{L{-}O{-}O^{\bullet} + L{-}H \rightarrow \underset{\text{lipid hydroperoxide}}{L{-}O{-}OH} + L^{\bullet}}$$

The propagating step in most free radical chain oxidations is transfer of a hydrogen atom from the organic substrate to a chain-carrying peroxyl radical. The rate of this process is usually slow as compared to other reactions in the chain, and the rate constant, $k_p$, appears in the overall rate expression.[21] The steady-state rate eq 7 is usually expressed as consumption of oxygen, L−H, or as the formation of peroxide products. In this rate expression, $R_i$ is the rate of radical generation, $k_p$ is the propagation rate constant, and $k_t$ is the termination rate constant for the reaction of two peroxyl radicals being converted to nonradical products.

$$-\frac{d[O]}{dt} = \frac{k_p}{(2k_t)^{1/2}}[L-H]R_i^{1/2} \quad (7)$$

Bimolecular H-atom transfer propagation rate constants, $k_p$, determined for lipid substrates range from less than 1 to over 2 × $10^3$ $M^{-1}$ $s^{-1}$.[23,46] These rate constants are lower than the rate

**Scheme 5. Peroxyl Radical Addition to Styrene and a Linoleate Conjugated Diene**




constants that have been determined for many other radical propagation reactions, a testament to the fact that the peroxyl radical is relatively stabilized and unreactive. The rate constants for autoxidation of dozens of organic compounds were determined over 40 years ago by Ingold and his collaborators using classical rotating sector techniques.[23,27] Indeed, it was the pioneering work by Ingold,[27] along with the earlier efforts of Mayo,[28] Walling,[29] and others, that provided the mechanistic framework for understanding the fundamentals of radical chain oxidation.

The kinetics of linoleate ester peroxidation has been studied in independent laboratories, and a $k_p$ for methyl linoleate of 60−62 $M^{-1}$ $s^{-1}$ is a consensus value at 30 °C.[23,68] Oleate, a monounsaturate has a measured $k_p$ of less than 1 $M^{-1}$ $s^{-1}$, while saturated substrates would be expected to have measurable rates only at temperatures above 60 °C.[20] Consistent with these propagation rate constants, linoleate readily undergoes peroxidation at 37 °C, while the reaction of oleate is sluggish and the saturated chain of palmitate esters is essentially inert to free radical oxidation at this temperature. Of the fatty acids and esters, it is the homoconjugated diene of the linoleates (18:2), linolenates (18:3), arachidonates (20:4), eicosapentaenotes (20:5), and docosahexaenotes (22:6) that are targets of free radical abstraction by propagating peroxyl radicals. Indeed, the relative rate of H-atom transfer propagation for linoleate and the higher polyunsaturates depends directly on the number of $CH_2$ centers that are flanked by two double bonds. Linoleate has one such center, arachidonate has 3, eicosapentaenoate has 4, and docosahexaenoate has 5, and the relative propagation rate constants determined for these compounds are in a ratio of 1, 3.2, 4.0, and 5.4 (Table 1).[46]

Cholesterol, a monounsaturated sterol lipid, is an order of magnitude more reactive than oleate, a monounsaturated fatty acid. Cyclic alkenes tend to be better hydrogen atom donors than acyclic alkenes, likely because the orientation of the reactive allylic C−H bond is such that maximum delocalization of radical character can be achieved in the transition state without significant distortion of the structure. In addition to geometry effects, the allyl radical generated by removal of the cholesterol C-7 hydrogen is trisubstituted by alkyl groups and thus more stabilized than the oleate-derived allyl radical that is only disubstituted. Cyclohexene, for example, has a $k_p$ of 5 $M^{-1}$ $s^{-1}$, oleate is less than 1 $M^{-1}$ $s^{-1}$, and cholesterol is 11 $M^{-1}$ $s^{-1}$. The rate constant determined for 7-DHC deserves particular comment. 7-DHC is an intermediate in the biosynthesis of cholesterol and Vitamin $D_3$, and it appears to be one of the most oxidizable lipids known that maintains a free radical oxidation chain reaction. The C−H bonds that are broken during chain propagation at C-9 and C-14 of the sterol (Figure 2) have torsion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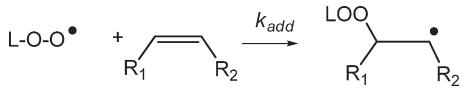
angles with the conjugated diene in ring B so that minimal structural change occurs in the transformation of precursor to delocalized carbon radical (Figure 3). The torsion angles are less favorable for the active hydrogens in 8-dehydrocholesterol, and the rate constant for H-atom transfer is consequently lower, even though the same pentadienyl radical is generated from both precursors.

$$\log[k_p/(M^{-1}\ s^{-1})] = 16.4 - 0.2\ \text{C}-\text{H BDE} \quad (8)$$

BDEs of the relevant C−H bond correlate directly with propagation rate constants. Thus, Korcek et al. reported that $\log(k_p)$ of the H-atom transfer reaction to secondary peroxyl radicals correlated with the BDE in kcal/mol of the C−H according to eq 8, where $k_p$ is given per active hydrogen at a site.[20] Recently, the rate constant of H-atom transfer has been correlated with the C−H BDE calculated by DFT methods by an equation analogous to eq 8 but with constants of 18.9 and 0.22.[44]

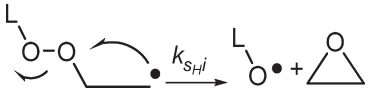
Oxygen radicals other than peroxyl can propagate a free radical chain by hydrogen atom abstraction. One such notable example of this is the transfer of an H-atom from a lipid such as linoleate to the tocopheroxyl radical in a sequence designated as tocopherol-mediated peroxidation or TMPO. Vitamin E is a generally good lipophilic antioxidant because the tocopheroxyl radical has low activity and can be removed by radical−radical reactions. However, in lipoprotein particles where such reactions are limited, it promotes lipid peroxidation unless it is scavenged by a secondary antioxidant such as ascorbate. Tocopherol-mediated peroxidation will be limited to situations in which radical−radical termination reactions are physically restricted such as in lipoprotein particles, and establishing the importance of this process in vivo will be challenging and perhaps impossible.[69]

### 1.2.3. Peroxyl Radical Addition.




Peroxyl free radicals undergo addition reactions to carbon−carbon double bonds. While this reaction has not been studied extensively in the context of lipid peroxidation, it is well established in organic free radical chemistry, with the copolymerization of styrene and oxygen being a well-established autoxidative process. In stryrene−oxygen copolymerization, peroxyl radicals add to the styrene alkene followed by addition of oxygen to the newly formed carbon radical, as shown in Scheme 5A.[70,71] The reaction continues to "grow" a polymer chain by alternate addition of styrene and oxygen until termination or other competing processes occurs. Most alkenes or dienes that undergo polymerization on their own will undergo addition reactions with peroxyl radicals. Conjugated dienes are particularly prone to addition by peroxyl radicals; butadiene and isoprene readily undergo copolymerization with oxygen.[72] Conjugated dienes are, of course, primary products in the oxidation of polyunsaturated fatty acids and esters, and products that are the result of subsequent peroxyl radical addition to the primary HPODE products of linoleate peroxidation also have been isolated; see Scheme 5B.[73] Rate constants for peroxyl radical addition have been determined for a number of substrates by classical techniques, and the constants determined depend on the resonance stabilization energy of the carbon radical formed upon addition.[74] Alkenes with isolated double bonds are much less prone to undergo addition reactions, and direct addition of peroxyl radicals to monounsaturated lipids is not generally observed with the exception of cholesterol (vide infra).

### 1.2.4. Intramolecular Radical Substitution on Peroxide.

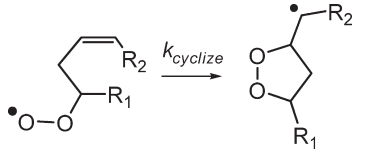



Carbon radicals formed by addition of a peroxyl radical to an alkene can undergo addition reactions with oxygen leading to copolymers as described in Scheme 5, but intramolecular homolytic substitution ($S_Hi$) on the peroxide bond is frequently observed to compete with oxygen addition. The styrene copolymer shown in Scheme 5 can "unzip", for example, in a sequence of reactions that is initiated by $S_Hi$attack of a carbon radical on peroxide, Scheme 6. Following this initial $S_Hi$ reaction, the copolymer essentially unravels by successive fragmentations of weak peroxide bonds and C−C bonds that are located $\beta$ to peroxides. Copolymerization of conjugated dienes with oxygen also gives rise to a polymer that "unzips" by $S_Hi$ reaction and subsequent polymer backbone.[72]

In the simplest version of the $S_Hi$, a carbon radical with a $\beta$-peroxide is converted to an alkoxy radical and an epoxide. Epoxides are frequently observed as products of free radical oxidation reactions, and undoubtedly some of these isolated epoxides are formed by the peroxyl radical addition and $S_Hi$ mechanism. Cholesterol, for example, can undergo epoxidation by the peroxyl radical addition $S_Hi$ mechanism as shown in Scheme 7.[26] In this transformation, epoxidation of cholesterol occurs in an addition reaction of a peroxy radical to the 5,6-double bond on the sterol, as shown in Scheme 7. Subsequent $S_Hi$ on the peroxide bond then leads to a lipid alkoxy radical and the epoxide. While this free radical mechanism undoubtedly occurs under some conditions, direct expoxidation of double bonds apparently can also occur without intervening free radicals.

Among lipid substrates, conjugated alkenes should be more susceptible to peroxyl radical addition chemistry than isolated olefins. Thus, conjugated linoleic acid should be much more prone to epoxidation than linoleic acid itself. Also, recent studies demonstrate that peroxyl radical addition to a conjugated double occurs in free radical oxidation of cardiolipin, a unique phospholipid with four linoleate chains in the same molecule.[75] Decomposition of the cross-linked peroxide bond generates an epoxide and 4-hydroxy-nonenal (4-HNE) (vide infra). By the same token, the conjugated diene sterol 7-DHC should form epoxide more readily than cholesterol; see section 3.2. In fact, epoxides have been found as products of peroxidation of both cholesterol and 7-DHC.

### 1.2.5. Peroxyl Radical Cyclization.




Radical cyclization reactions are responsible for the generation of cyclic peroxides when polyunsaturated lipids undergo peroxidation reactions. Arachidonic acid or its esters are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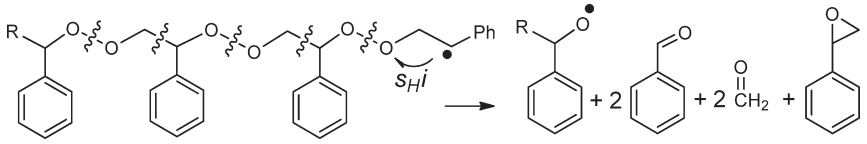
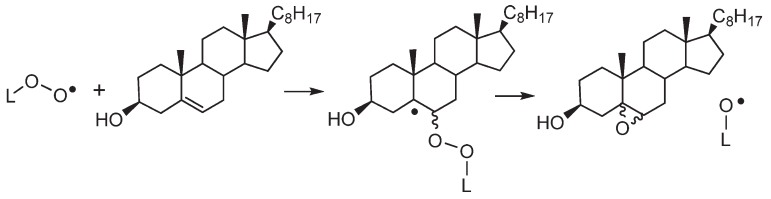
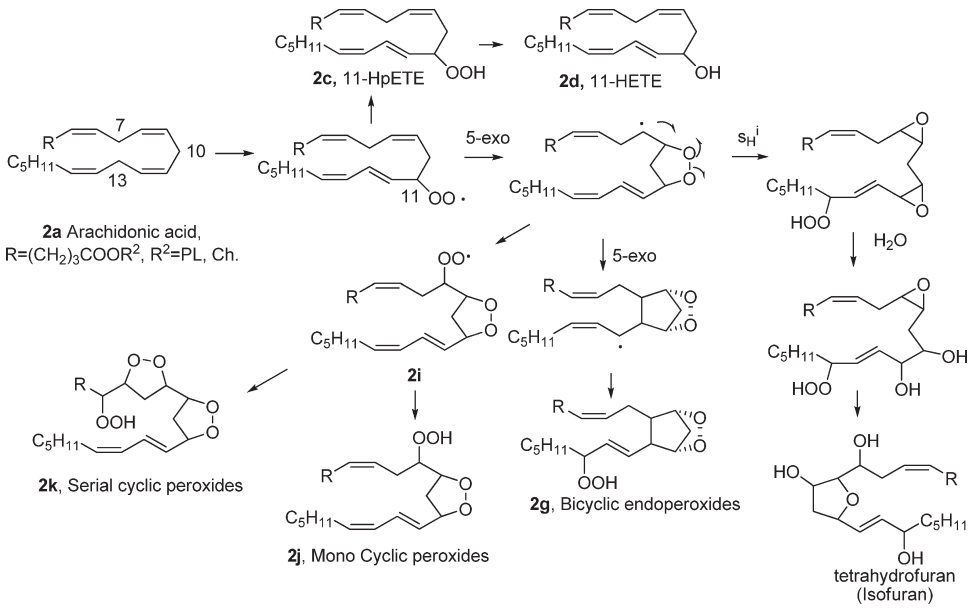
**Scheme 6. $S_Hi$ Initiated Unzipping of Styrene−Oxygen Copolymer**



**Scheme 7. Epoxidation of Cholesterol by Addition−Homolytic Substitution**



**Scheme 8. Major Pathways of Free Radical Oxidation of Arachidonic Acid**[a]




[a] Shown only for the 11-HPETE radical for simplicity.

important lipid substrates for cyclooxygenase and lipoxygenase enzymes, and the arachidonate free radical oxidation products, the isoprostanes, have been used extensively as biomarkers for lipid peroxidation in vivo.[15] Arachidonate is more susceptible to oxidation than is linoleate because it has three bisallylic positions that are possible sites for initial hydrogen atom abstraction. In the presence of good hydrogen atom donors, six major hydroperoxide products, the hydroperoxyeicosatetraenoates (HPETEs), are obtained (Scheme 8).

In contrast to the linoleate hydroperoxide products (HPODEs), the major HPETE products and the reduced analogue hydroxyeicosatetraenoate products (HETEs) of arachidonate oxidation have *Z,E*-conjugated diene. Little or none of the corresponding *E,E* arachidonate products are observed, while these can be the major products of linoleate oxidation. The *E,E* linoleate hydroperoxides are formed by a peroxyl radical fragmentation oxygen readdition mechanism as described in Schemes 2−4 with the critical $\beta$-fragmentation rate constant for the linoleate being about 700 $s^{-1}$. For arachidonate and any unsaturated fatty acid or ester having three or more double bonds, peroxyl radical cyclization to a five-membered ring can occur. The rate constant for cyclization has not been rigorously established, but estimates of 500−1000 $s^{-1}$ allow cyclization to compete with $\beta$-fragmentation to the *E,E* diene structure.[49] Therefore, little of the *E,E* products are observed.

In the absence of hydrogen atom donors, peroxidation of arachidonate gives more complex product mixtures. Consider, for example, the reaction pathways available for a peroxyl radical formed from H-atom abstraction from arachidonic acid,


dx.doi.org/10.1021/cr200084z |*Chem. Rev.* 2011, 111, 5944–5972

Scheme 8.[76−78] In this scheme, one of the six arachidonate peroxyl radicals, the 11-HPETE precursor, has multiple reaction pathways that open to it subsequent to a peroxyl radical cyclization.[79] Free radicals tend to prefer cyclizations to give five-membered rings, identified as 5-*exo* in the scheme, and peroxyl radicals are no exception. The carbon radical formed upon cyclization has several subsequent mechanistic branch points available. It can itself undergo a 5-*exo* cyclization on to the conjugated diene leading ultimately to a bicyclic endoperoxide identified in the scheme as an isoprostane endoperoxide. While the carbon radical can also undergo $S_Hi$ attack giving rise to an epoxy alcohol, it can also add oxygen, forming a new peroxyl radical that has most of the same mechanistic opportunities available to it that were available to the initial peroxyl radical in the sequence. When one of the epoxides is hydrolyzed, the hydroxyl group attacks the remaining epoxide to form a tetrahydrofuran-containing compound with a number of regio- and diastereomers. These compounds were given a trivial name as Isofuran.[80] A mechanistic study using $^{18}O$ labeled oxygen or water showed incorporation of two $^{18}O$ from oxygen gas and one $^{18}O$ from water, consistent with the proposed mechanism.[80] Serial cyclic peroxides and monocyclic peroxides can thus be generated by these established mechanistic pathways.[81]

Shown in Scheme 8 are the primary free radical lipid peroxidation products. Most of these compounds have limited stability and can be further converted to other products. A great effort has been devoted to study the formation of the bicyclic endoperoxides and their downstream products because these compounds with structures closely related to the prostaglandins are generated.[76,82] The latter are generated from cyclooxygenases and regulate many aspects of human physiology and pathology. The formation of analogous compounds of the prostaglandins, termed isoprostanes (IsoPs), represents a completely different pathway from the enzymatic mechanism, and this radical mechanism has important biological relevance.[83,84] The isoprostane compounds are a complex mixture of regio- and diastereoisomers, whereas the prostaglandins are generated enzymatically in only one enantiomeric form. Furthermore, isoprostanes are formed primarily on esterified phospholipids, and they are subsequently hydrolyzed by phospholipases, while prostaglandins are formed from free arachidonic acid that is released from the phospholipids enzymatically upon stimulation.[85,86]

### 1.3. Peroxidation of Membrane Lipids

Free radical oxidation of phospholipids in lipid membrane (lipid bilayer or liposome) follows the same kinetic rate law as in homogeneous systems (eq 7).[87−93] The oxidizability of lipid in membrane, however, is much lower than that in homogeneous solution due to slower diffusion rate within the membrane and the proposed "floating peroxyl radical hypothesis", diffusion of the polar peroxyl radical to the polar surface region.[87,88,92]

Peroxidation in lipid bilayers has been studied, and the H-atom transfer rate constants from different donors in lipid membrane have been reported.[46] The concentration of an H-atom donor is difficult to define in a lipid bilayer due to the variable density of the membrane having different phospholipid, sterol, and other biomolecule compositions. This problem was solved by using mole fraction ($n$) to represent the H-donor concentration. Mole fraction is defined as the ratio between the moles of the H-atom donor that is under study and the total moles of acyl chains, sterols, and other additives (such as α-tocopherol) in the lipid mixture. The product ratio of

**Table 2. Bimolecular Rate Constants for Hydrogen Atom Transfer Propagation of Lipid Substrates at 37 °C in Liposomes**

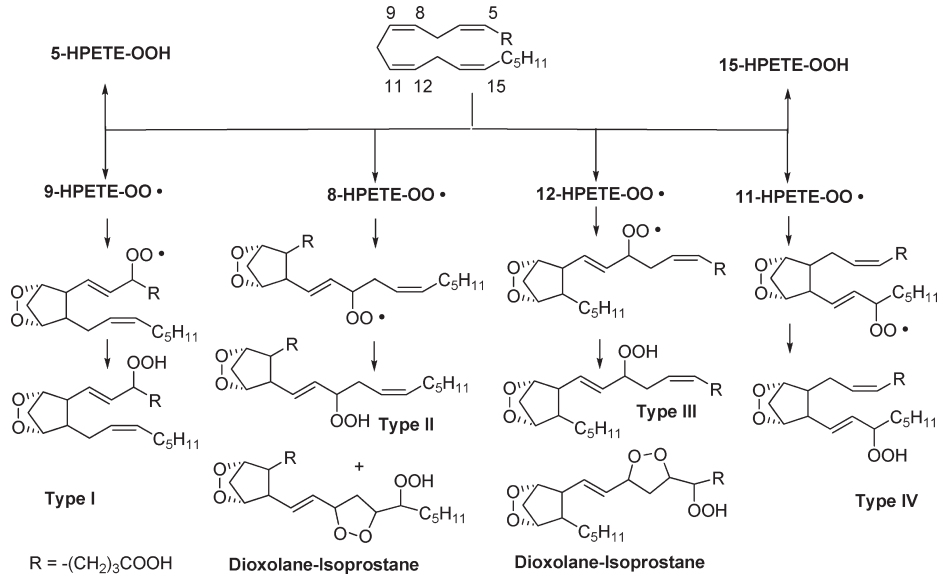
| H-donor | $k_p$ in liposome ($n^{-1}$ $s^{-1}$) |
|---|---|
| linoleate | 35[a] |
| arachidonate | 115 ± 7[b] |
| eicosapentaenoate | 145 ± 8[b] |
| docosahexaenoate | 172 ± 13[b] |
| 7-DHC | 832 ± 86[b] |

[a] Antunes, F., Barclay, L.; et al. *Int. J. Chem. Kinet.* **1998**, *30*, 753−767. [b] Xu, L; Davis, T.; Porter, N. *J. Am. Chem. Soc.* **2009**, *131*, 13037−13044.

*Z,E/E,E*-HODEs increases linearly with the concentration of added H-atom donors, confirming the similar kinetic behavior of lipid peroxidation in the bilayer as compared to that in solution. The rate constants in lipid bilayers determined in this way are shown in Table 2. The relative reactivities of linoleate (18:2), arachidonate (20:4), eicosapentaenoate (20:5), and docosahexaenoate (22:6) in lipid bilayers are 1:3.3:4.1:4.9, a ratio that is remarkably similar to their relative reactivities in solution (1:3.2:4.0:5.4, Table 1).[46] While 7-DHC is still the most reactive lipid in membrane among those studied, its relative reactivity to linoleate as an H-atom donor is less in lipid bilayer (24 times more reactive than linoleate) than in solution (62 times more reactive). The reason for this observation may lie in that 7-DHC does not diffuse as freely in a bilayer (laterally or medially) due to its rigid structure. Barclay and co-workers reported similar changes in reactivities of antioxidants from solution to membrane and cited a combination of reasons for the differences.[91,94] For example, BHT (Table 1, $k_p = 3.2 \times 10^3$ $M^{-1}$ $s^{-1}$) is several orders of magnitude less reactive than α-tocopherol ($k_p = 3.5 \times 10^6$ $M^{-1}$ $s^{-1}$) at 37 °C in solution,[95−97] but they have almost the same activity in membrane.[91]

Although the overall kinetic expression in bilayers is the same as in solution, the product distribution is somewhat different.[46] For examples, the kinetic behavior of two tautomeric *Z,E/E,E*-HODE pairs (9-*Z,E*/13-*E,E* and 13-*Z,E*/9-*E,E*) appear to be different in liposome, while those product ratios are essentially the same in solution. Two reasons for the difference in liposomes and isotropic media have been offered. First, a polarity difference of the local bilayer environment leads to different rate constants of β-fragmentation ($k_\beta$) of the 9- and 13-peroxyl radicals. The $k_\beta$ of peroxyl radicals is known to decrease in polar solvents and with hydrogen bonding,[98,99] and the experimental results seem to indicate that 9-peroxyl radicals have a smaller $k_\beta$ than do the 13-peroxyls. This is consistent with the fact that 9-peroxyl radicals are closer to the polar aqueous interface. A second possible effect for free radical oxidation in liposomes being different from that in solution is the fact that the relative propagation rate constants ($k_p$) of a particular H-atom donor toward 9- and 13-peroxyl radicals may be different. For example, it appears that 7-DHC is a much better H-atom donor to 9-peroxyl radicals than to 13-peroxyl radicals. These differences in $k_p$ and $k_\beta$ also led to differences in total amounts of 9- and 13-hydroperoxides formed. In fact, more 9-HODEs than 13-HODEs were observed in all of the clocking experiments in liposome except those in the presence of docosahexaenoate.[46] Docosahexaenoate may be a better H-atom donor to 13-peroxyl radical due to the location of the reactive bisallylic methylene groups.

**Scheme 9. Regiochemistry of Isoprostanes Formation from Arachidonic Acid Oxidation**




On the basis of the same kinetic rate law and comparable relative reactivities (to linoleate) of H-atom donors in membrane and in solution, it is reasonable to propose that the peroxidation mechanism (see previous sections) will be similar in lipid membrane to that in solution. However, due to pronounced local environmental effects, a primary product distribution may be expected in membranes that is different from that observed in isotropic media.

## 2. FREE RADICAL OXIDATION OF POLYUNSATURATED LIPIDS

### 2.1. Stereochemical Course of Isoprostane Formation

The biosynthesis of the prostaglandin family of compounds has been extensively explored.[100] The enzymatic formation of prostaglandin endoperoxides is believed to be the result of a controlled 5-*exo* peroxyl radical cyclization reaction of the lipid peroxyl radical onto the arachidonate backbone. The COX enzyme apparently uses standard free radical chemistry, but the enzyme controls stereochemistry of the process leading to only one of hundreds of possible products that can be formed by the uncontrolled free radical chain oxidation of arachidonic acid. Efforts have been made to understand the regio- and diastereochemistry of isoprostanes because these different steroisomers may have different biochemistry and physiological effects and they may form in different amounts under different biological circumstances.[101−103] Six hydroperoxyl radicals are generated from the free radical oxidation of arachidonate. These peroxyl radicals undergo cyclization reactions as shown in Scheme 8 to generate four sets regioisomers of isoprostanes. As shown in Scheme 9, 9- and 11-peroxyl radicals cyclize to form the 5- and 15-series of $G_2$-IsoPs, respectively, whereas the 8- and 12-peroxyl radicals give rise to the 12- and 8-series of isoprostanes. The two terminal peroxyl radicals at the 5- and 15-positions cannot undergo direct cyclization, but $\beta$-fragmentation of these two peroxyl radicals and oxygen readdition can occur to form the 9- and 11-peroxyl radicals.[77]

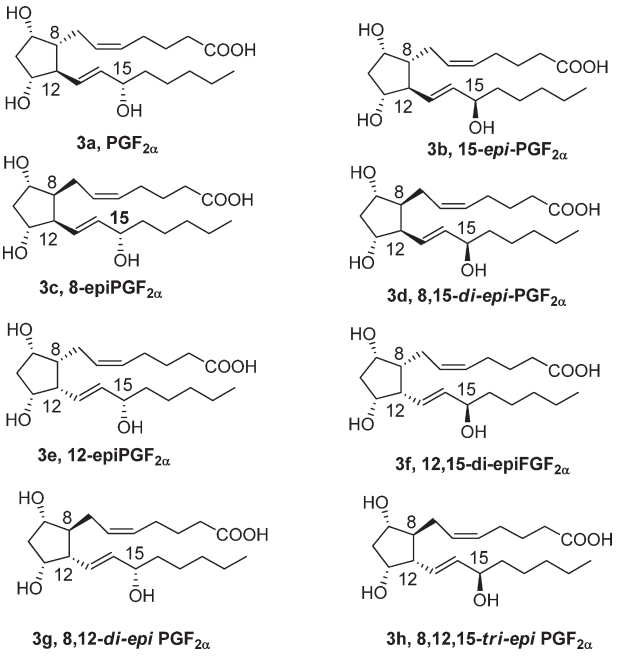



**Figure 6.** Stereoisomers of the 15-series isoprostanes.

The four sets of isoprostane regioisomers are not formed equally, but the 5- and 15-series are generally observed to be at least an order of magnitude more abundant than the 8- and 12-series.[103] Studies have shown that the cyclized peroxyl radicals derived from 8- and 12-HPETE can undergo further 5-*exo* cyclization to form a novel series of isoprostane, dioxolane-isoprostanes. The same chemistry can be applied to predict the regioselectivity of isoprostane-like compounds derived from EPA and DHA.[104−106]

### 2.2. Diastereoselectivity of Isoprostanes Formed in Free Radical Oxidation

Within each class of the $G_2$-isoprostanes, eight pairs of racemic diastereomers can be formed because five stereogenic centers are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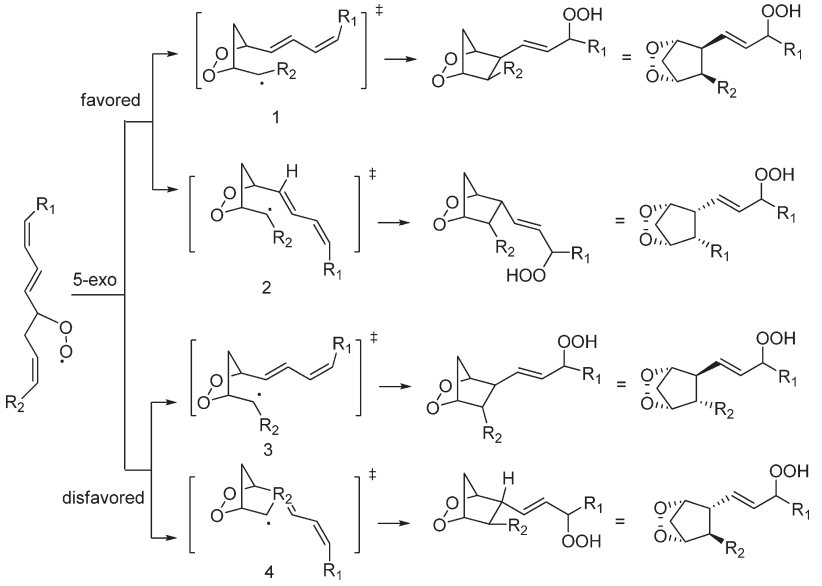
**Scheme 10. Transition States That Control the Stereochemistry of 5-*exo* Cyclization**




produced in the two 5-*exo* cyclizations and the two oxygen additions (Figure 6, shown as $F_2$-isoprostanes). Constraints on product stereochemistry include the fact that the peroxide bond in the bicyclic moiety must be in a *cis*-configuration for bicyclization to occur and the fact that the free radical process, by definition, generates a racemic mixture.

Diastereoselectivity of isoprostane formation was studied by analysis of the $F_2$-IsoPs due to the limited stability of $G_2$-IsoPs and to simplify the oxidation profile. Furthermore, the product distribution could be simplified by examining only those products that form starting from a specific peroxyl radical derived from an isolated precursor hydroperoxide. Thus, starting from the 11-hydroperoxide, only the 15-series isoprostanes are formed. All eight diastereoisomers of the 15-series isoprostanes, as TMS ether derivatives, have been separated and identified by GC−MS and compared to independently synthesized standards.[82]

Isoprostanes with *cis*-substituted ring side chains are formed preferentially to those that have *trans*-substitution; that is, **3d**, **3c**, **3e**, and **3f** are major diastereomers as compared to **3a**, **3b**, **3g**, and **3h**. One of the diastereomers coelutes with prostaglandin $PGF_{2\alpha}$. The product stereoselectivity can be understood on the basis of peroxyl radical 5-*exo* cyclization via a Beckwith−Houk transition state.[107,108] Transition states **1** and **2** are energetically favored because the two side chains are in the equatorial positions, whereas transition **3** and **4** has one of the side chains in axial orientation (Scheme 10). It is noteworthy that the natural prostaglandins have *trans*- configurations.

The difference in energies between the two sets of transition states has been calculated to be only 3.4 kcal/mol at 37 °C, and thus a significant amount of $F_2$-IsoPs with *trans* side chains is formed by a nonenzymatic pathway. In fact, the enantiomer of the enzymatic product, *ent*-$PGF_{2\alpha}$ is the major $F_2$-IsoPs found in human urine, and this enantiomer must be derived from the nonenzymatic free radical pathway.[109]

The 11- and 15-peroxyl radicals both generate 15-series isoprostanes by the mechanism shown in Scheme 10. The conjugated diene between C-12 and C-15 has *E*,*Z* geometery in the 11-peroxyl radical formed directly from 11-HPETE, whereas the 11-peroxyl radical generated from 15-HPETE (from $\beta$-fragment and oxygen readdition) has *E*,*E* geometry (Scheme 11)[82]

When the oxidation reaction was carried out starting from either the 11- or the 15-HPETE esters, the same isoprostane products were formed with only a marginally different selectivity (Scheme 11).[82] The product geometry apparently translates directly from allyl radical geometry to the configuration at the C15 epimeric center.

### 2.3. Dioxetane Mechanism for IsoP Formation

Scheme 11 shows that the 15-HPETE peroxyl radical does not directly generate isoprostanes, but after $\beta$-fragmentation and oxygen readdition it forms an 11-peroxyl radical that can cyclize to give the 15-series isoprostanes. An alternative mechanism that involves a dioxetane intermediate to explain the formation of IsoPs from 15-HPETE has been proposed,[110] and this mechanism has been suggested to account for in vivo generation of isoprostanes.[111,112] In this mechanism, the 15-peroxyl radical undergoes a 4-*exo* cyclization followed by oxygen addition and two 5-*exo* cyclizations. The bicyclic endoperoxide is formed by the fragmentation of the dioxetane intermediate.

In the dioxetane mechanism, the stereochemistry at C15 should be preserved according to Scheme 12, but in the $\beta$-fragmentation mechanism oxygen loss and re-addition would lead to loss of configuration at C15. When an optically pure 15-HPETE ester was subject to free radical oxidation conditions, the resulting $F_2$-IsoPs were shown to contain a completely racemic mixture of 15-series isoprostanes.[77] This experiment is consistent with the $\beta$-fragmentation and oxygen readdition mechanism, and it suggests that the dioxetane mechanism is unlikely.

### 2.4. Factors Affecting Arachidonate Oxidation Product Distribution

As shown in Scheme 8, autoxidation of fatty acids containing three or more double bonds may generate a number of different oxidation products. There are branching points in the pathways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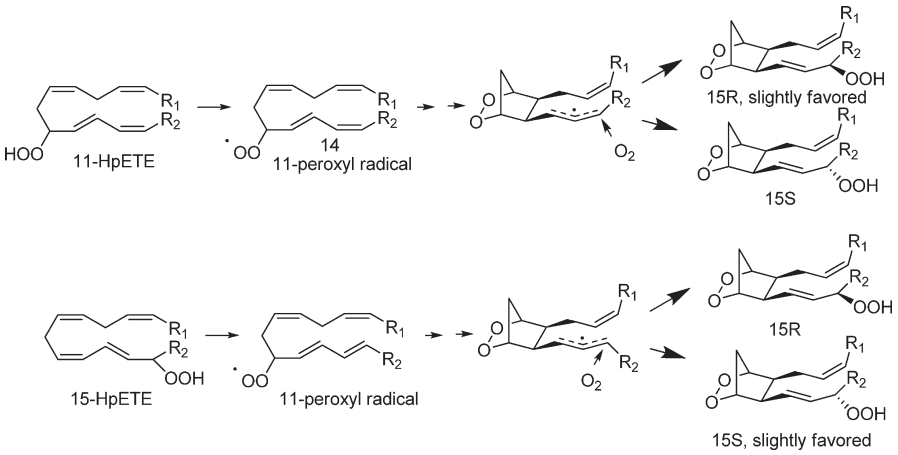
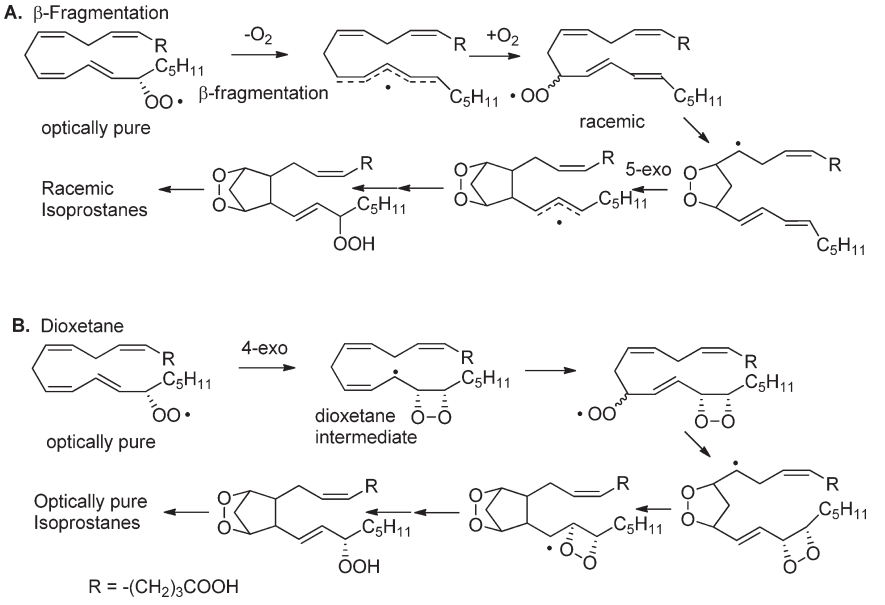
**Scheme 11. Two Mechanisms for 15-Series Isoprostane Formation**




**Scheme 12. Free Radical Mechanisms for Formation of Isoprostanes from 15-HPETE: (a) β-Fragmentation−Oxygen Addition, (b) Dioxetane Intermediate**




that determine the distribution of different oxidation products, the two most important factors being the local reducing environment and oxygen tension. In the presence of high concentration of good hydrogen atom donors, peroxyl radicals are readily trapped to form hydroperoxides. Thus, the formation of cyclization products including isoprostanes, monocyclic peroxides, serial cyclic peroxides, and isofurans is suppressed.

When the peroxyl radical is not trapped and undergoes cyclization, oxygen tension may become the dominant factor that determines the subsequent product distribution. The atmosphere contains around 20% oxygen. In humans, the gradient of oxygen tension (partial pressure) decreases from 150 mmHg in the environment, to approximately 110 mmHg in the lungs, to 95 mmHg in the arterial system, down to only 30 mmHg in most tissues.[1] In the pathways for IsoP formation, the second 5-*exo* cyclization involves a carbon-centered radical. Thus, oxygen addition competes with this cyclization reaction. The former is a second-order reaction and oxygen tension is the determining factor, while the carbon-centered radical cyclization is a first-order reaction. The observation that $F_2$-IsoPs are formed in vivo in significant amount reflects the fact that 5-*exo* cyclization in vivo competes with the direct oxygen addition due to the low oxygen tension. The rate constant for oxygen addition is diffusion controlled ($10^8$ or $10^9$ $mol^{-1}$ $s^{-1}$), whereas the rate constant for carbon-centered radical 5-*exo* cyclization or $S_Hi$ is around $10^5$ $s^{-1}$ in model systems.[113,114] Thus, micromolar to submillimolar concentrations of oxygen in a biological locale make the two pathways competitive.

Formation of isofurans in vivo appears to be favored under high oxygen tension.[115] It is conceivable that high oxygen tension would lead to the formation of monocyclic and serial cyclic peroxides, and the monocyclic peroxides have been suggested as precursors for isofurans.[80] Homolytic cleavage of the cyclic peroxide bond generates two alkoxyl radicals. One of the alkoxyl radical undergoes 3-*exo* cyclization onto the conjugated double bonds to form an epoxide and hydroperoxide. The other alkoxyl


dx.doi.org/10.1021/cr200084z |*Chem. Rev.* 2011, 111, 5944–5972

radical may abstract a hydrogen atom to form an alcohol. The hydroxyl group attacks the epoxide to produce a tetrahydrofuran compound, and the isofuran is obtained upon reduction (Scheme 13). However, the mechanism seems to be unlikely because, on the basis of this mechanism, all four oxygen atoms incorporated into isofuran would be from an oxygen molecule, while labeling experiments using $^{18}O_2$ and $H_2O^{18}$ indicated that one of the oxygen atoms originated from water. Thus, experimental evidence seems to be consistent with the pathway illustrated in Scheme 8, that is, the formation of diepoxide by $S_Hi$.

### 2.5. Conversion of Bicyclic Endoperoxides ($G_2$-IsoPs) to Other Isoprostanes

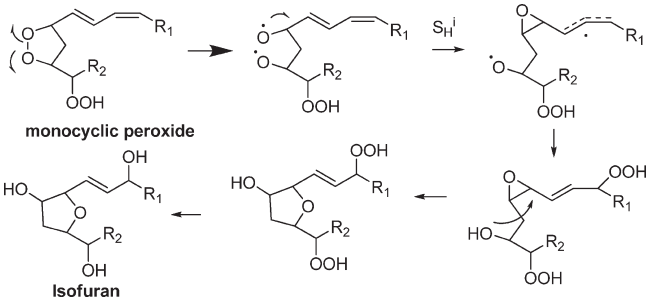
The formation of $G_2$-IsoPs represents one of the major pathways of arachidonic acid or ester autoxidation, but the $G_2$-IsoPs are extremely labile due the presence of bicyclic endoperoxide moiety. These species therefore serve as precursor to downstream decomposition products.[84] The major pathways for endoperoxide decomposition are summarized in Scheme 14. The $F_2$-IsoPs, produced from the reduction of the bicyclic endoperoxides and the hydroperoxide, are relatively stable, and the levels of these compounds have become a reliable marker for oxidative stress in vivo. The bicyclic endoperoxide moiety readily undergoes rearrangement to form $D_2/E_2$-IsoPs, and further dehydration leads to $J_2/A_2$-IsoPs compounds, respectively.[116,117] Loss of a malondialdehyde (MDA) from the endoperoxide gives rise to a heptadecatrienoic acid in a process similar to that observed for $PGG_2$. Isolevuglandins (Isoketal) are another class of major products of the endoperoxides, and the $\gamma$-ketoaldehyde motif in these compounds makes them extremely reactive.[118,119] The epoxide-IsoPs have been identified by Berliner et al. from the oxidation of 1-palmitoyl-2-arachidonyl-phosphotidylcholine (PAPC) in LDL, and it represents one of the most biologically active compounds in LDL oxidation.[120,121] In addition to isoprostanes, compounds analogous to the thromboxanes and leukotrienes have also been identified in free radical oxidations.

The $F_2$-IsoPs have received attention as biomarkers for oxidative stress because they do represent a significant fraction of the products formed in controlled oxidation of arachidonate esters.[51] The yields of isoprostanes from oxidation of arachidonic acid originally reported were very low, less than 0.1%, but the conditions of oxidation have a dramatic effect on the products actually isolated from an oxidation reaction because the peroxide intermediates are unstable to acids, bases, reducing metals, and other reducing agents such as thiols. Thus, determination of the yield of isoprostanes after a 24 h oxidation of arachidonic acid in pH 7 buffer with ROS-generating enzymes understandably leads to low isoprostane product yields. On the other hand, if controlled oxidations of arachidonate in an organic solvent at low conversion are examined, and the product yield is calculated on the basis of consumed arachidonate, then yields of isoprostanes determined are in the range of 15−20%.[51] One assumes that a free radical oxidation of arachidonate in vivo would also give yields of this magnitude, but the initially formed peroxides are very unstable in water, and reducing agents and catabolic enzymes will have their effects. Indeed, the identification and analysis of the isoprostanes as biomarkers of oxidative stress is all the more remarkable because these compounds are apparently no more than the detritus of free radical oxidation products that survive at detectable levels.

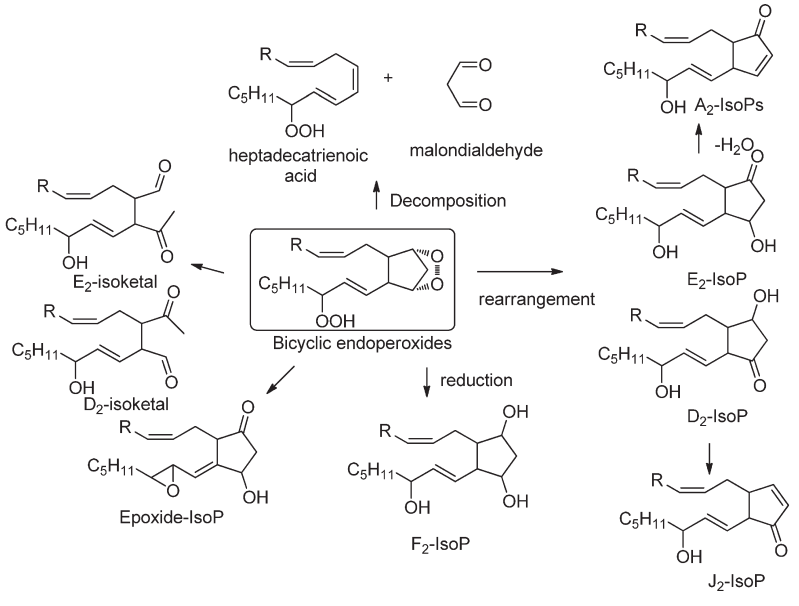
### 2.6. 4-HNE and Other Fragmentation Products of Oxidized Lipids

In addition to the cyclization oxidation products like the isoprostanes, fatty ester lipid hydroperoxides also undergo fragmentation

**Scheme 13. Monocyclic Peroxides as Precursors to Isofurans**




**Scheme 14. Conversion of Bicyclic Endoperoxides ($G_2$-IsoPs) to Downstream Products**





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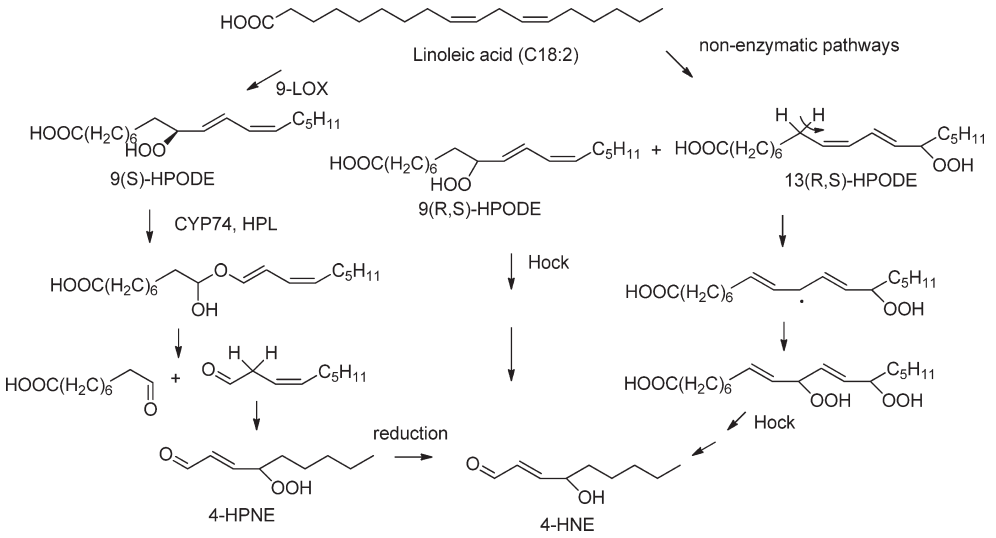
**Scheme 15. Proposed Chemical Mechanisms of 4-HNE Formation from Linoleic Acid**




reactions to produce truncated oxidation products.[122] Not only can these products be generated from oxidation of free fatty acids including arachidonic acid and linoleneic acid, but phospholipid esters also form significant amounts of these truncated oxidation products. In fact, it has been suggested that 1 equiv of $F_2$-IsoP is generated from consumption of 130 000 equiv of arachidonic acid by the in vitro oxidation of rat liver microsomes. In the same experiment, the ratio of malondialdehyde/$F_2$−-IsoP is approximately 34 000:1.[123] It should be noted, however, that in vitro microsome experiments have little to do with the yield of isoprostanes formed in the primary step of arachidonic acid oxidation. Indeed, careful book-keeping experiments show that conversion of arachidonate to isoprostanes can occur with yields as high as 30%.[51] The low yield of product formed in the microsomal studies must result from the fact that subsequent reactions divert the isoprostane endoperoxide to secondary byproducts.

4-HNE and MDA are the two most-studied lipid electrophiles generated from lipid peroxidation.[124,125] In human plasma, the concentration of free $F_2$-IsoPs is around 0.14 nM (50 pg/mL plasma), while 4-HNE is in the micromolar ($\mu$M) to millimolar (mM) range. 4-HNE mediates a wide variety of biological processes from DNA damage and mutagenesis, inflammatory response, cell growth, to apoptosis.[124] Over the past two decades, 4-HNE has become one of the most studied reactive lipid electrophiles.[124−128] As a highly reactive $\alpha,\beta$-unsaturated aldehyde, 4-HNE can form adducts with DNA, proteins, and amine-containing lipids due to its strong electrophilic character.[129−131] 4-HNE has also been involved in multiple signaling events including antioxidant response (Keap1/Nrf2 pathways), heat shock response,[132] ER stress, stress-responsive MAP kinase signaling, NF-$\kappa$B signaling, and DNA damage response signaling.[127]

The chemical mechanism for the formation of 4-HNE has been studied extensively.[133] The general consensus is that 4-HNE is generated from lipid hydroperoxides of $\omega$-6 fatty acids including linoleic acid and arachidonic acid. As shown in Scheme 15, linoleic acid can be oxidized to 9(S)-HPODE by plant lipoxygenase, and a plant cytochrome P450 CYP74C (hydroperoxide lyase, HDL) cleaves the hydroperoxide and generates two aldehydes through a Hock-type reaction. The 3Z-nonenal is readily oxidized to 4-HPNE by a subsequent nonenzymatic pathway. Even though the analogous hydroperoxide lyase cleavage of lipid hydroperoxides have not been found in animals, similar pathways were proposed to account for the formation of 4-HNE from 9- and 13-HPODE. Chiral hydroperoxides, 9($S$)- and 13($S$)-HODES, were used as model substrates to test the hypothesis. 4-HPNE derived from 9($S$)-HOPDE was racemic, consistent with the Hock cleavage mechanism. In contrast, 4-HPNE from 13($S$)-HPODE largely retained the $S$-configuration from the starting hydroperoxide.[134] An alternative dihydroperoxide intermediate was proposed to account for this observation. This 10,13-dihydroperoxide may be formed from the hydrogen atom abstraction at C8, followed by molecular oxygen addition at C10. However, the synthesized dihydroperoxide did not form 4-HPNE under autoxidative conditions. Thus, the formation of 4-HNE from 9-HPODE may undergo Hock cleavage, whereas from 13-HPODE, an alternative mechanism must exist.

Vitamin C has been shown to catalyze the formation of 4-HNE and other lipid aldehydes via a proposed pathway involving the hydroperoxide-derived alkoxyl radical.[135] Ferrous iron ($Fe^{2+}$) has been shown to be involved in the formation of 4-HNE from decomposition of α-hydroperoxy endoperoxides.[136] A recent study demonstrated that 4-HNE was formed from heme-catalyzed decomposition of a diendoperoxide derived from 5-LOX and COX-2 pathways.[137] However, the fatty acid hydroperoxy endoperoxide and diendoperoxide intermediates can only be formed from fatty acids containing at least three double bonds. A revision to the mechanisms for 4-HNE formation stemmed from the observation of epoxy-alcohol and epoxy-hydroperoxide from the autoxidation of 15($S$)-HETE and 15($S$)-HPETE. The chirality of the hydroxyl- or hydroperoxyl group is also retained.[138] These observations suggested a potential mechanistic connection between the formation of the epoxides and 4-HNE, which may involve cross-molecular peroxyl radical reactions. The peroxyl radical addition and decomposition have been well documented in styrene−oxygen copolymerization as shown in Schemes 5 and 6.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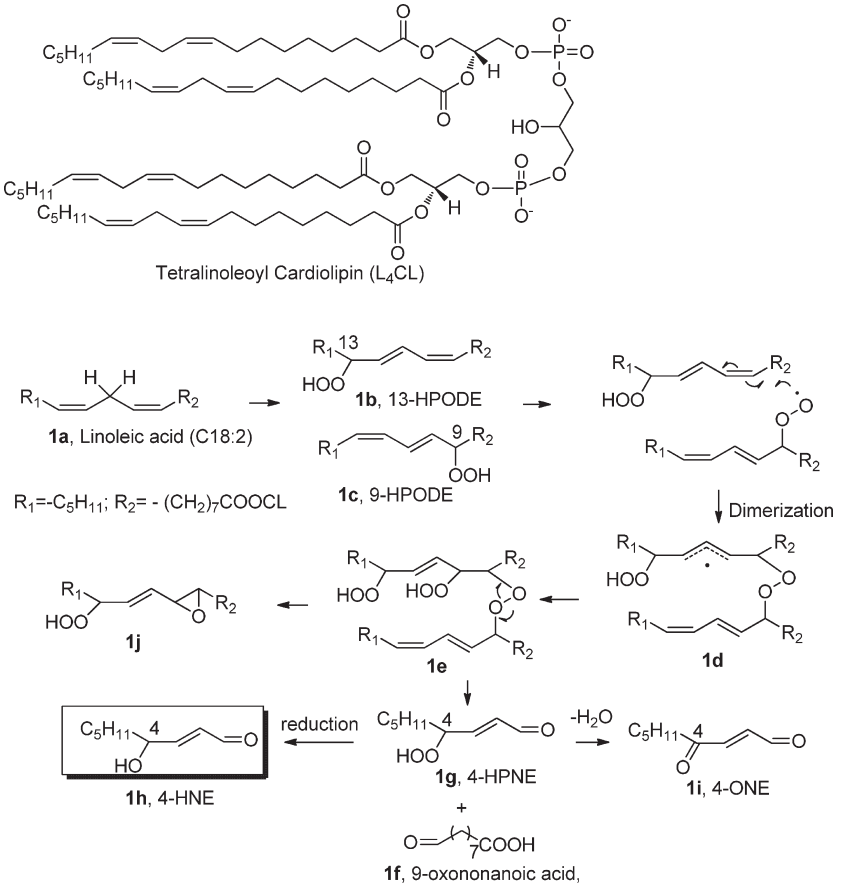
**Scheme 16. Peroxyl Radical Addition Mechanism for 4-HNE Formation**




Furthermore, formation of 4-H(P)NE from dimerization of methyl linoleate has been suggested.[139] However, the biological relevance of this mechanism for the 4-HNE formation from cellular membrane phospholipids has not been explored until our recent studies demonstrates that a significant amount of 4-HNE forms from free radical oxidation of cardiolipin, a unique phospholipid with four linoleate chains in the same molecule (Scheme 16).[75] The critical intermediates that are consistent with the proposed mechanism have been identified and characterized. Some of these intermediates appear to be formed in rat liver tissue under carbon tetrachloride-induced lipid peroxidation, a prototypical model for oxidative stress where 4-HNE was originally identified.[140] The formation of 4-HNE via this novel mechanism has significant biological relevance because tetralinoleoylcardiolipin is the major cardiolipin in most mammalian cells and its association with cytochrome *c* to elicit a peroxidase activity, and subsequent oxidation plays a critical role in apoptosis.[75] This mechanism has implications in intrinsic pathways of apoptosis and other functions of mitochondria.

In addition to the free electrophiles such as 4-HNE and MDA, the truncated oxidation products that are still bound to phospholipids have attracted increasing interest.[141] Some of the truncated phospholipids derived from linoleoyl, arachidonyl, and DHA-containing PC and PS play a major role in macrophage recognition and phagocytosis via scavenger receptor CD36. Hazen et al. made important efforts to identify the potent CD36 ligands (shown in Figure 7) that may have significant implication in innate immunity and thrombosis.[122,142] These oxidized phospholipids exist at micromolar concentration in plasma, and they increase 4−30-fold in mice fed a hypercholesterolemic diet. Thus, platelet CD36 links hyperlipidemia, oxidative stress, and a prothrombotic phenotype to these oxidized phospholipids.

## 3. FREE RADICAL OXIDATION OF STEROLS

Sterols with double bonds in the ring are subject to free radical oxidation due to the weak bond dissociation energy of the allylic C−H bonds and low entropy demand in forming the transition state (see section 1).[143] Cholesterol free radical oxidation and the oxidation products, that is, oxysterols, have been implicated in pathologies such as atherosclerosis,[144] Alzheimer's disease,[11,145] retinal degeneration,[146−150] and metabolic disorders such as Niemann−Pick disease.[13]

Recently, 7-DHC was reported to be the most reactive lipid molecule yet studied toward free radical oxidation.[46] In a typical free radical oxidation reaction in organic solution, 7-DHC reacts with a rate constant (2260 $M^{-1}$ $s^{-1}$) that is 200 times that of cholesterol (11 $M^{-1}$ $s^{-1}$), and about 20 times that of arachidonic acid (197 $M^{-1}$ $s^{-1}$), a highly reactive PUFA that is discussed in previous sections (Table 1).[46] Some 15 oxysterols were identified in the reaction mixture of 7-DHC free radical oxidation in solution, with many more oxidation products still not yet characterized.[59] These findings opened a new pathway for oxysterol generation in vivo, especially in cholesterol biosynthesis disorders such as the Smith−Lemli−Opitz syndrome (SLOS)[151−155] and X-linked dominant chondrodysplasia punctata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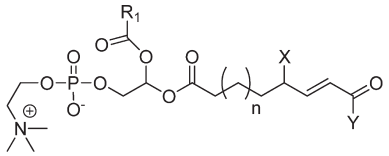

PAPC, n = 1, $R_1$=-$C_{15}H_{31}$ HODiA-PC, X=OH, Y=OH KODiA-PC, X=O, Y=OH HOOA-PC, X=OH, Y=H KOOA-PC, X=O, Y=H

PLPC, n = 5, $R_1$=-$C_{15}H_{31}$ HDdiA-PC, X=OH, Y=OH KDdiA-PC, X=O, Y=OH HODA-PC, X=OH, Y=H KODA-PC, X=O, Y=H

**Figure 7.** Representative structures of oxidized PC as CD36 ligands.

(CDPX2),[156−159] cholesterol metabolic disorders like cerebrotendinous xanthomatosis (CTX),[160,161] and breast cancer,[162] all of them being diseases with elevated levels of 7-DHC in fluids and/or tissues of patients. 7-DHC free radical oxidation and its oxysterols may also play important pathophysiological roles in the development of skin-related diseases as the level of 7-DHC is relatively high in human skin, where 7-DHC is converted to pre-Vitamin $D_3$ upon UV irradiation.[163] In the plasma of a normal individual, 7-DHC is present at a level that is one twenty thousandth to one thousandth that of cholesterol.[164,165] On the basis of these values and given the same biological environment and conditions of radical initiation, oxysterols generated from 7-DHC will contribute as much as 1−20% to the total oxysterol pool in plasma based on its propagation rate constant as an H-donor (Table 1), a relevant number even for a normal individual. As levels of 7-DHC and antioxidant capacity may vary in different tissues, contribution from 7-DHC to the overall oxysterol level will vary accordingly.

Biological activities of autoxidation-derived oxysterols and those formed from enzymatic reactions have been reviewed in great detail.[166−169] Here, we focus on the free radical oxidation mechanism of cholesterol and 7-DHC. The reaction mechanism of 8-DHC will also be discussed because it is closely related to that of 7-DHC. Although the oxidation mechanism of cholesterol is complicated, 7-DHC and 8-DHC provide many more mechanistic options for free radical attack and further transformations. Oxidation by other oxidants such as singlet oxygen ($^1O_2$) and ozone ($O_3$) is discussed briefly to compare with the free radical oxidation product profile.

### 3.1. Cholesterol Oxidation

**3.1.1. Free Radical Oxidation.** Free radical reaction of a cholesterol molecule can be initiated through either H-atom transfer from the allylic position ($k_H$) or peroxyl radical addition ($k_{add}$) to the double bond, and both types of reactions propagate a radical chain (section 1).

Initial hydrogen atom abstraction by a peroxyl free radical occurs by donation of a hydrogen atom from the sterol C-7 position.[170] ESR studies confirmed that C-7 allylic radical is the only detected radical formed at room temperature[171] and C-7-oxysterols are the most frequently observed and abundant oxysterols generated from free radical oxidation.[144,164,172,173] Abstraction of one of C-7 hydrogen atoms results in an allylic radical that has two resonance contributors (Scheme 17). Although both C-5 and C-7 in Scheme 17 can be trapped by oxygen, only C-7 products have been observed, probably because $\beta$-fragmentation from the 5-peroxyl radical is rapid as compared to propagation pathways.[44] The presence of a very reactive hydrogen donor (R-H) should trap a C-5 peroxyl radical. This expectation is based on analogy for the formation of 11-HPODE from linoleate. 11-HPODE is normally observed at very low levels, or it is nondetectable during free radical oxidation of linoleate, but it can be readily observed when good hydrogen donors, such as α-tocopherol, are present during the oxidation reaction.[55] A detailed discussion of the $\beta$-fragmentation mechanism can be found in section 1.

Ground-state oxygen (triplet) will add to the radical at the C-7 position on either the α or $\beta$ face of ring-B of cholesterol, and this results in formation of both 7α- or 7$\beta$-hydroperoxycholesterol (7α- and 7$\beta$-OOH-Chol). Reduction of the hydroperoxides by reducing agents in the biological environment, for example, peroxidase and $Fe^{2+}$,[174,175] gives 7α- and 7$\beta$-hydroxycholesterol (7α- and 7$\beta$-OH-Chol). Disproportionation of 7α- and 7$\beta$-OOH-Chol will also give 7α- and 7$\beta$-OH-Chol along with 7-ketocholesterol (7-keto),[170] one of the most abundant oxysterols found in atherosclerotic lesions.[144] 7-Keto can also be formed from 7α- and 7$\beta$-OOH-Chol by dehydration[176] and by the "Russell mechanism" where a radical chain termination reaction occurs by the reaction of two peroxyl radicals followed by the decomposition of the resulting tetroxide.[22]

Hydrogen atoms at C-4 of cholesterol are also potential targets for H-atom abstraction. 4$\beta$-Hydroxycholesterol (4$\beta$-OH-Chol) has been identified in vitro and in vivo, including in rat liver, human plasma, and food.[177−179] In addition, both 4α- and 4$\beta$-OH-Chol have been identified at comparable levels in the in vitro oxidation of low-density lipoprotein and in human atherosclerotic plaques, suggesting an autoxidation mechanism in these cases (Scheme 18).[180] The mechanism for oxidation at C-4 is essentially the same as the H-7 mechanism.

Some of the known oxysterols shown in Scheme 18 may be formed from 6-OOH-Chol by a mechanism that is different from those proposed in the literature.[170] It is known that 4$\beta$-OH-Chol, but not 4α-OH-Chol, can be formed from cholesterol by cytrochrome P450 3A4 (CYP3A4).[181,182] The fact that 4α-OH-Chol has only been observed in limited cases suggests that the H-4 mechanism only plays a minor role in the free radical oxidation of cholesterol. Further evidence is still needed to elucidate whether both 4α- and 4$\beta$-OH-Chol can be formed in the free radical oxidation of cholesterol.

Peroxyl radical addition to the double bond on ring-B of cholesterol leads to another set of products. Primarily, cholesterol 5α,6α- and 5$\beta$,6$\beta$-epoxides are formed via a presumed $S_Hi$ mechanism that follows peroxyl radical addition (Scheme 7). The byproduct, an alkoxyl radical, will carry on the radical chain by abstracting another hydrogen atom from the environment. Cholesterol 5,6-epoxides seem to be two of the major products observed in free radical oxidation of cholesterol, but the reported ratios of the α and $\beta$-epoxides are variable in the different systems studied.[25,183−185] There is some evidence that the 5α,6α- and/or 5$\beta$,6$\beta$-epoxy cholesterols can be formed enzymatically in bovine liver microsomes and adrenal cortex,[185,186] but no specific enzyme has been shown to catalyze the epoxidation of cholesterol.

Both α- and $\beta$-epoxides can undergo further transformation by cholesterol epoxide hydrolase to give cholesta-3$\beta$, 5α,6$\beta$-triol,[186,187] which can be further oxidized to 3$\beta$, 5α-dihydroxy-cholestan-6-one (DHCAO) (Scheme 19). The oxidizing agent for conversion of the triol to DHCAO has not been identified in vivo, but it could be a combination of several oxidants, including air, $Fe^{3+}$, or enzymes. DHCAO has been identified as a metabolite of 5,6-epoxides in rat liver subcellular


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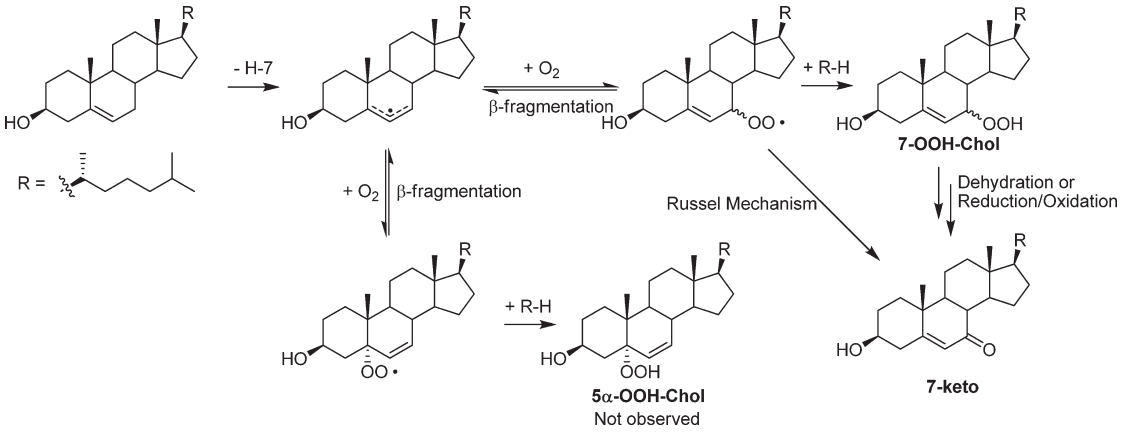
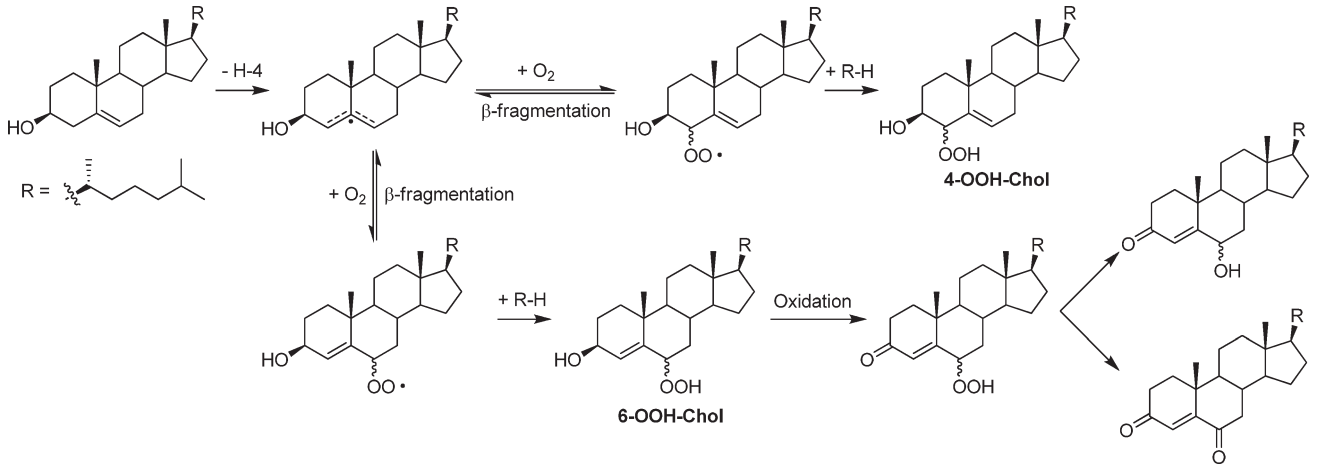
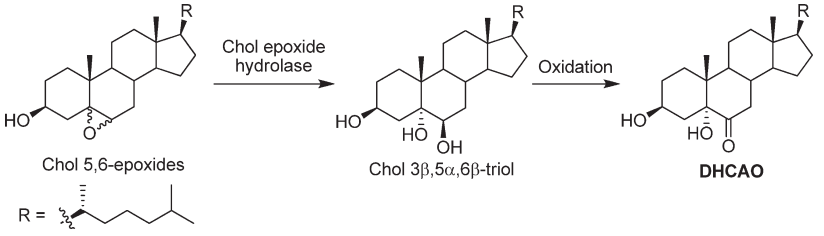
**Scheme 17. Free Radical Oxidation of Cholesterol via Hydrogen Atom Abstraction at C-7 (H-7 Mechanism)**




**Scheme 18. Free Radical Oxidation of Cholesterol via Hydrogen Atom Abstraction at C-4 (H-4 Mechanism)**




**Scheme 19. Biological Transformation of Chol 5,6-Epoxides**




fractions,[188] and it has been found in pulmonary epithelial cells that are exposed to ozone.[189−191] DHCAO is toxic to a human bronchial epithelial cell line, and it inhibits cholesterol biosynthesis in the submicromolar range in the same cell line.[189]

**3.1.2. Oxidation by Other Oxidants.** Cholesterol oxidations by singlet oxygen and ozone have been extensively studied, and both give rise to distinct product profiles. Singlet oxygen ($^1O_2$) oxidation of cholesterol gives 5α-hydroperoxycholesterol (5α-OOH-Chol) as the major product, and 6α- and 6β-OOH-Chol as minor products.[170,192,193] 5α-OOH-Chol rearranges to 7α-OOH-Chol preferentially via a peroxyl radical intermediate upon homolytic cleavage of the ROOH bond without incorporation of isotope-labeled oxygen from the environment (Scheme 20).[58] Experimental and computational studies support the contention that a solvent cage effect prevents the diffusion of the allyl radical-oxygen pair formed from β-fragmentation of the 5-peroxyl radical,[194] resulting in preferential addition of the oxygen to the 7α-position. Subsequently, the 7α-peroxyl radical can epimerize to 7β-peroxyl radical with incorporation of the atmospheric oxygen, suggesting formation of a free allylic radical.[58]

Ozonolysis of cholesterol in aqueous dispersion, solution, and in biological samples gives unique products such as 5,6-secosterol ketoaldehyde and its aldol condensation product resulting from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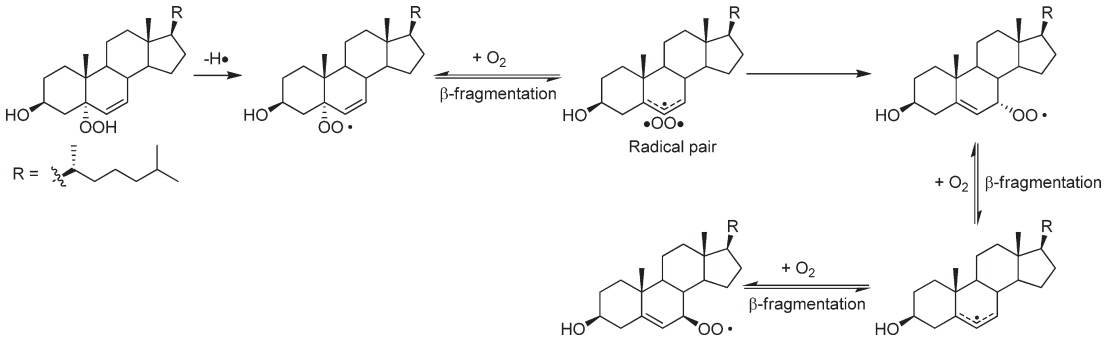
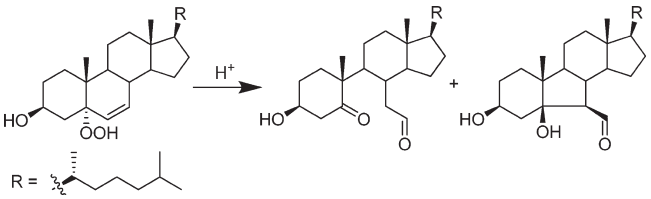
**Scheme 20. Rearrangement of 5α-OOH-Chol to 7α-OOH-Chol**




**Scheme 21. Hock Fragmentation of 5α-OOH-Chol**




C5═C6 double bond cleavage.[189,190,195−197] In addition, some oxysterol products similar to those formed from free radical oxidation were observed, for example, cholesterol 5α,6α- and 5β,6β-epoxides, 3β,5α,6β-triol, and DHCAO, in aqueous dispersion and solution.[189] Cholesterol 5β,6β-epoxide is observed as the major product during ozonolysis of cholesterol in lung surfactant instead of the ring-opening products,[189] probably because of the different reaction environment or the fact that reactive aldehydes form adducts with proteins or other nucleophiles and therefore cannot be isolated. It is also noteworthy that decomposition of the $^1O_2$ oxidation product, 5α-OOH-Chol, via Hock fragmentation can give rise to the 5,6-secosterol ketoaldehyde and its aldol condensation product (Scheme 21).[198]

### 3.2. 7-DHC and 8-DHC Oxidation

**3.2.1. Free Radical Oxidation.** 7-DHC has the highest measured propagation rate constant ($k_p$) known for any lipid toward free radical chain oxidation at 2260 $M^{-1}$ $s^{-1}$.[46] A comparison of rate constants with other oxidizable lipid molecules is shown in Tables 1 and 2. There are four allylic hydrogen atoms in a 7-DHC molecule. However, molecular mechanics calculations suggest that the reactive hydrogen atoms are at H-9 and H-14, both of them being well positioned for abstraction by peroxyl radicals, with the dihedral angles of C(7)−C(8)−C(9)−H(9) and C(7)−C(8)−C(14)−H(14) being close to 90°.[46] The axial β-hydrogen atom on C-4 is apparently shielded by the axial C-19, while the abstraction of the equatorial α-hydrogen atom is not kinetically favored. The product profile of 7-DHC free radical oxidation also suggests that the active hydrogen atoms are at positions H-9 or H-14 of the sterol. With only two reactive hydrogen atoms, the propagation rate constant per hydrogen atom is approximately 1130 $M^{-1}$ $s^{-1}$ on average for 7-DHC. 1,3-Cyclohexadiene, a simple analogue of 7-DHC, has a per hydrogen $k_p$ of 55 $M^{-1}$ $s^{-1}$,[23] some 20 times lower than that of 7-DHC. 1,4-Cyclohexadiene, which is generally considered to be an excellent H-atom donor, has a rate constant of 66 $M^{-1}$ $s^{-1}$ per hydrogen atom,[95] a rate constant that is still substantially lower than that for 7-DHC but higher than that of the bis-allylic C−H bond in linoleate (31 $M^{-1}$ $s^{-1}$ per hydrogen atom) and the simply allylic C−H in oleate.[44,143,199,200]

The availability of two highly reactive hydrogen atoms in 7-DHC and the two pentadienyl radicals formed from hydrogen atom transfer provide more mechanistic options for chemistry from 7-DHC relative to that from cholesterol. For purposes of discussion, the 7-DHC free radical oxidation mechanism can be divided into two separate pathways, designated the H-9 and H-14 mechanisms. A combination of the basic free radical reactions (see section 1), that is, oxygen addition to carbon-centered radicals, peroxyl radical 5-*exo* cyclization, intermolecular peroxyl radical addition, and intramolecular radical substitution on the peroxide bond ($S_Hi$), provide a rationale for understanding most of the products observed. A detailed description of the reaction mechanism has been reported.[59]

In either the H-9 or the H-14 mechanism, initial abstraction of the hydrogen atom on C-9 or C-14 gives a pentadienyl radical, which could lead to peroxyl radicals at C-5, C-7, and C-9 or at C-5, C-7, and C-14 (Figure 5). In either case, only in the presence of a very good H-atom donor such as α-tocopherol would the kinetically labile 7-peroxyl radical be trapped to give 7-OOH-5,8(9)-diene or 7-OOH-5,8(14)-diene. The β-fragmentation of the bis-allylic 7-peroxyl radical would be expected to be fast ($2.6 \times 10^6$ $s^{-1}$ for the linear analogue[95]).

In the H-9 mechanism (Scheme 22), 5-*exo* cyclization of both the C-5 and C-9 peroxyl radical would give the same endoperoxy allylic radical, which could undergo $S_Hi$ reaction to give an epoxide or the addition of oxygen at C6 or C8. Peroxyl radicals thus formed from oxygen addition could undergo further transformations such as hydroperoxyl radical elimination to give the conjugated diene. The rate constant for 5-*exo* cyclization of 5- or 9- peroxyl radical is not known, but a similar cyclization reaction in a linear system has been estimated to be ca. 800 $s^{-1}$.[49] If a similar rate constant holds for the 5- or 9- peroxyl radical, a moderately good H-atom donor should be able to compete with the cyclization pathway and generate the dienyl hydroperoxides at the expense of cyclic peroxides formed.

In the H-14 mechanism (Scheme 23), the C-5 or C-14 peroxyl radical does not have a favorable structural configuration for a 5-*exo* cyclization, and thus the dienyl hydroperoxides are the primary products formed from H-14 abstraction. Either one of these hydroperoxides can undergo further transformation due to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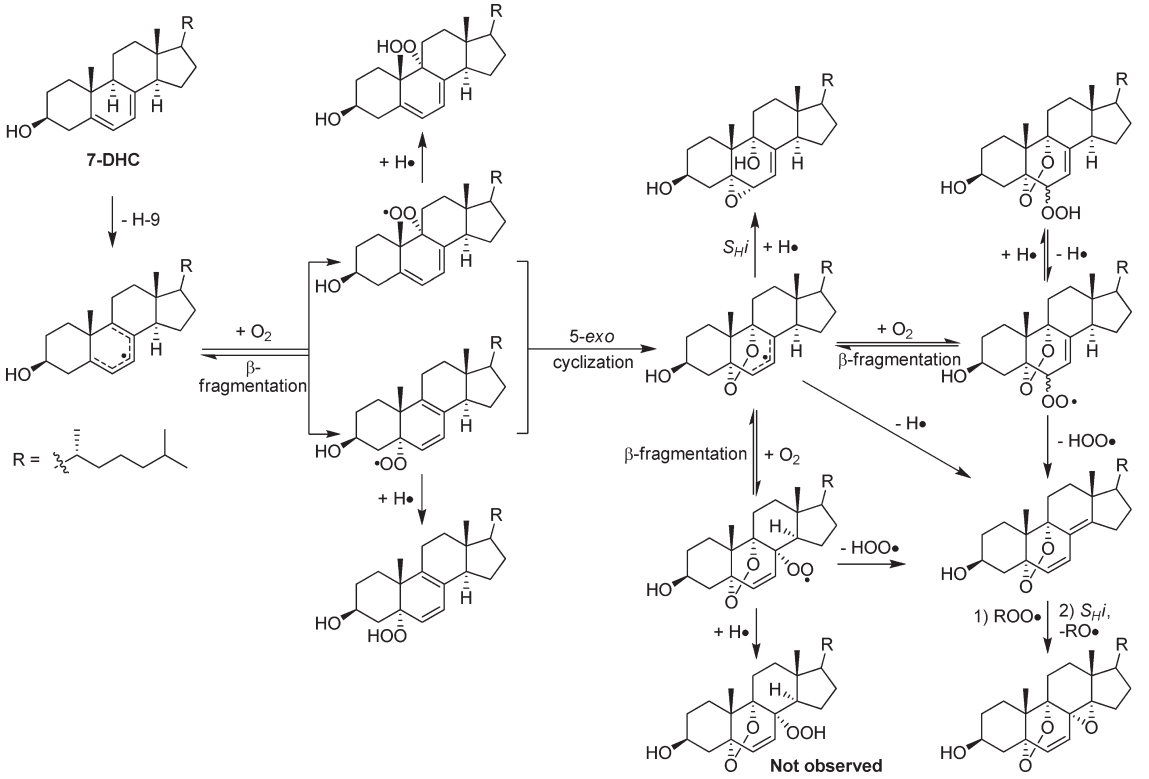
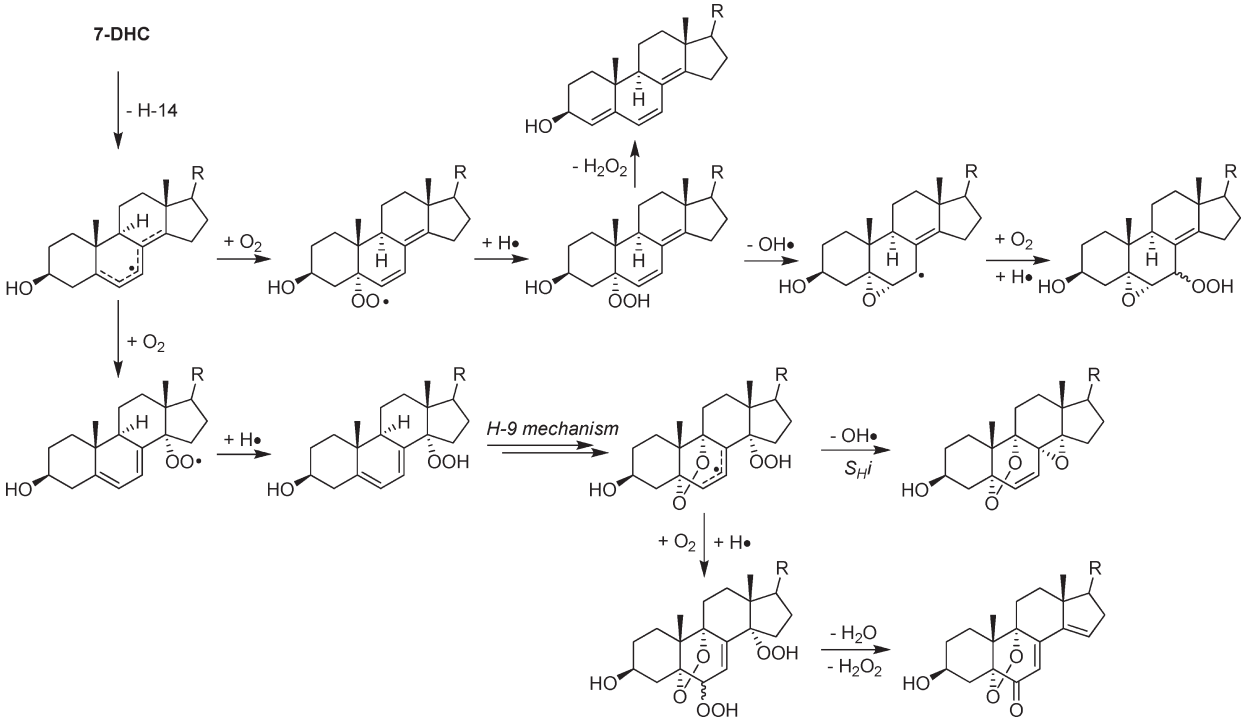
**Scheme 22. H-9 Mechanism for Free Radical Oxidation of 7-DHC**




**Scheme 23. H-14 Mechanism of Free Radical Oxidation of 7-DHC**




the remaining reactivity within the molecule. Notably, an epoxy endoperoxide can be formed from either H-9 or H-14 mechanism.

The conjugated diene of 7-DHC is also apparently prone to undergo intermolecular peroxyl radical addition, which results in a relatively stable allylic radical (Scheme 24). Subsequent $S_Hi$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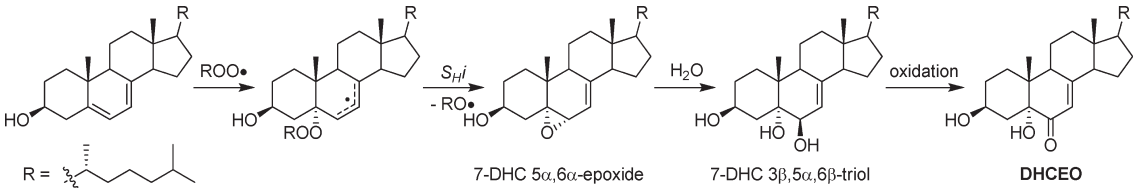
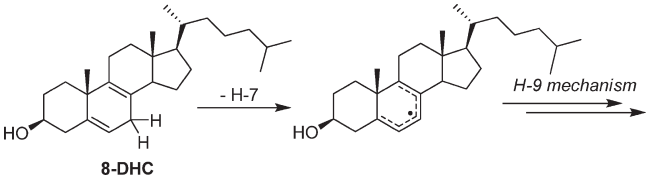
**Scheme 24. Free Radical Oxidation of 7-DHC by Peroxyl Radical Addition**




**Scheme 25. Free Radical Oxidation of 8-DHC**




reaction on the peroxide bond would give 7-DHC 5α,6α-epoxide as the product.[201] Ring-opening of the 5α,6α-epoxide followed by allylic oxidation would lead to 3β,5α-dihydroxycholest-7-en-6-one (DHCEO), an analogue of DHCAO from cholesterol. In fact, DHCEO has been reported to be a minor product of free radical oxidation of 7-DHC in solution and one of the major oxysterols observed in cell and animal models for SLOS, and it is a biomarker for 7-DHC peroxidation.[59,201,202]

Free radical oxidation of 8-DHC is expected to follow the H-9 mechanism of 7-DHC oxidation because the same pentadienyl radical will be formed after the removal of an H-atom at C-7 (Scheme 25). 8-DHC is also highly oxidizable with a propagation rate constant of 990 $M^{-1}$ $s^{-1}$, and its free radical oxidation indeed gives a spectrum of products that are similar to those formed from the H-9 mechanism of 7-DHC oxidation (Table 2; Xu, L.; Porter, N., unpublished results). 8-DHC is the other key cholesterol precursor that is elevated in SLOS patients, and it is also detected at high level in CDPX2 patients due to the enzymatic defect in $\Delta^8,\Delta^7$-isomerase that isomerizes the double bond at C-8 to C-7.[156−159]

**3.2.2. Oxidation by Other Oxidants.** Singlet oxygen [$^1O_2$] oxidation of 7-DHC has been studied carefully as has a similar transformation been studied for ergosterol, an analogue of 7-DHC present in plants.[203−205] $^1O_2$ oxidation of 7-DHC mainly gave the "ene" addition product, 7-OOH-5,8(9)-diene, and the 5,8-endoperoxide [4 + 2] cycloaddition product (Scheme 26). In addition, the 5,7,9(11)-triene is observed as a decomposition product of the primary peroxides. The 5,8-endoperoxide and the 7-hydroperoxide were reported to form in a ratio consistently determined to be 3:1.[203,204] The 5,7,9(11)-triene has been identified in plasma of a SLOS patients, and it has been suggested to be involved in the UV-A photosensitivity of SLOS patients.[206,207]

### 3.3. Free Radical Oxidation of Sterol Esters

Cholesterol fatty esters are major constituents of low-density lipoprotein, they are subject to free radical oxidation, and this process may play an important role in the development of atherosclerosis.[4] Cholesterol esters of saturated fatty acids may increase the oxidation rate of cholesterol due to their increased diffusion rate relative to cholesterol in a membrane bilayer, but polyunsaturated fatty esters may have other effects on sterol oxidation as well. In solution, PUFAs oxidize 6−30 times faster than cholesterol depending on the number of bis-allylic methylene groups (Table 1).[46] This suggests that the PUFA moieties of cholesterol esters will be oxidized in preference to the sterol nucleus. In fact, cholesterol exists largely as the linoleate ester (18:2) in human LDL, and linoleate is about 6 times more susceptible to peroxyl radical attack than is cholesterol. On the other hand, one expects that for 7-DHC fatty esters oxidization would occur primarily on the 7-DHC framework because 7-DHC oxidizes an order of magnitude faster in solution and in liposome than PUFAs (Tables 1 and 2).[46]

## 4. ANALYSIS OF LIPID OXIDATION MIXTURES

A plethora of oxidation products are generated from free radical lipid peroxidation, and some of the oxidation products, the $F_2$-isoprostanes, have reasonable stability in tissues and biological fluids and can be employed to provide a measure of oxidative stress status.[84] Most of the oxidation products, however, have limited stability, and they either decompose to form downstream metabolites or react with biomolecules including proteins, lipids, and nucleic acids to form adducts. Thus, the detection and quantification of these various oxidized lipids in vivo inevitably poses a significant challenge. Analysis of lipid peroxidation products and the study of their roles in biology has been an active area for decades, and various methods have been developed to assess lipid peroxidation. Historically, measurement of conjugated diene of lipid hydroperoxides such as the HODEs and HETEs by UV at 234 nm has been used to assess lipid peroxidation.[208] However, the application of this method has been limited due to the complication of interfering compounds having the same UV absorption. Light emission during hydroperoxide-induced oxidation of luminol has also been used as a chemiluminescence assay to assess lipid peroxidation.[209] This sensitive method combined with an HPLC separation has been used to analyze levels of hydroperoxides from cholesterol esters and phospholipids in plasma samples. The thiobarbituric acid reactive substance (TBARS) assay is one of the most frequently used methods to assess lipid peroxidation. The test is usually performed by heating a lipid peroxidation mixture and TBA in an acidic medium to form a red pigment, which shows an absorption maximum at 532 nm (with a molar extinction coefficient of $1.56 \times 10^5$) and fluorescence emission at 553 nm. The ferrous oxidation of xylenol (FOX) assay was developed by Wolff et al.[210] to detect the hydroperoxides formed from lipid peroxidation. This method is based on the fact that hydroperoxides oxidize ferrous (iron II) to ferric (iron III), and the resulting ferric ion can bind to xylenol orange to produce a colored complex with a strong absorbance at 560 nm ($\varepsilon = 4.3 \times 10^4$ $M^{-1}$ $cm^{-1}$ for $H_2O_2$ and *t*-butyl hydroperoxide).[210] Both the TBARS and the FOX assays are nonspecific methods, and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**Scheme 26. Oxidation of 7-DHC by $^1O_2$**

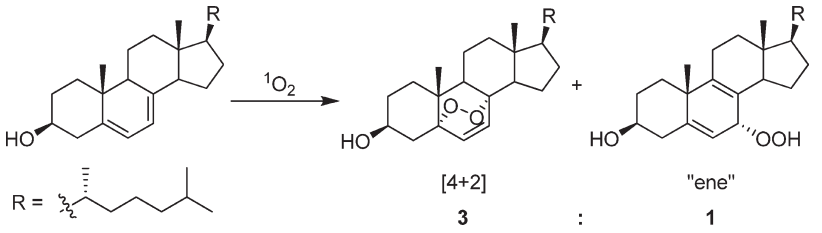
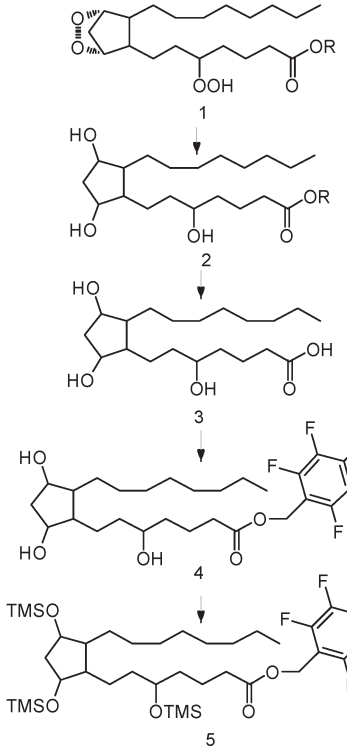



**Table 3. Analysis of Isoprostanes by Various MS Techniques**

| RepresentitativeLipid peroxidation products | MS methods |
|---|---|
|  | Ag+-CIS MS<br>ESI-Iontrap MS<br>ESI-Triple Quad MS<br>APCI-MS<br>GC-MS |

caution is recommended when these assays are used for the analyses of oxidized biomolecules other than lipid hydroperoxides, such as endoperoxides.[211]

Mass spectrometry (MS) coupled with other separation techniques has played an indispensible role in the detection and quantitation of lipid peroxidation products due to its superior sensitivity and specificity.[81,212,213] Since the development of soft ionization techniques in the late 1980s, the field of biological MS has witnessed an exponential growth.[214] These soft ionization methods that allow direct ionization of intact biomolecules such as proteins and lipids include electrospray (ESI), fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), and matrix-assisted laser desorption/ionization (MALDI).

We focus here on the MS techniques that have been developed to study the oxidation products of lipids. Oxidized lipids represent only a fraction of the lipid pool in a biological source, and thus it is challenging to detect these minor lipid mediators by most MS approaches. Yet in the last few decades, a number of MS-based approaches to study the oxidized lipid have been developed. Taking isoprostanes as an example, the major MS strategies used to characterize and quantify these biologically active lipid mediators are shown in Table 3.

### 4.1. GC−MS

Since the discovery of $F_2$-IsoPs in the early 1990s, measurement of these compounds by GC−MS has evolved as the "gold standard" to assess oxidative stress status in vivo.[215] Chemical ionization in the negative ion mode coupled with GC separation has been widely used to analyze these compounds due to its superior sensitivity and specificity.[216,217] However, a number of separation and chemical derivatization steps are required before these compounds are amenable for GC-MS analysis, and the assay is cumbersome and laborious. It nevertheless has the advantage of great sensitivity.

### 4.2. $Ag^+$ Coordination Ionspray (CIS)−MS

As shown in Table 3, bicycle endoperoxides are the first-formed isoprostanes that are precursor to the other classes of isoprostanes. Direct characterization of these endoperoxides is difficult, but it may also prove essential to study the biological roles and chemical mechanisms for the formation of these important lipids. The bicyclic endoperoxide moiety is extremely labile in water, but the compounds are stable in organic solvents and they likely survive in the hydrophobic core of lipoproteins.[82] Their presence in oxidized LDL may be of relevance to the initiation and formation of atherosclerotic plaque.

Characterization of these intact bicyclic endoperoxides as cholesteryl esters is difficult to undertake by conventional MS methods. Electrospray ionization (ESI) and APCI are widely used soft ionization modes and may be coupled efficiently to powerful separation techniques such as HPLC for the characterization of complex mixtures. Nonetheless, cholesteryl esters are highly lipophilic and lack ionization sites with the sufficient proton affinity required for conventional ESI and APCI applications.

Bayer et al. reported the use of a coordinating reagent to ionize highly lipophilic compounds, such as terpenes, sugars, aromatics, and vitamins.[218] A variety of coordinating reagents have been used to coordinate with hydrophobic analytes and make them suitable for MS analysis through a standard ESI−MS interface in a technique termed coordination ionspray mass spectrometry (CIS−MS). Silver ion has been used extensively for CIS−MS applications because of its ability to coordinate with double bonds or aromatics. Furthermore, silver ion adducts can be easily identified in a MS spectrum due to the characteristic doublet isotopic pattern of $[M + Ag^{107}]^+$ and $[M + Ag^{109}]^+$, because the natural isotopic abundance of silver is 52:48. Last but not least,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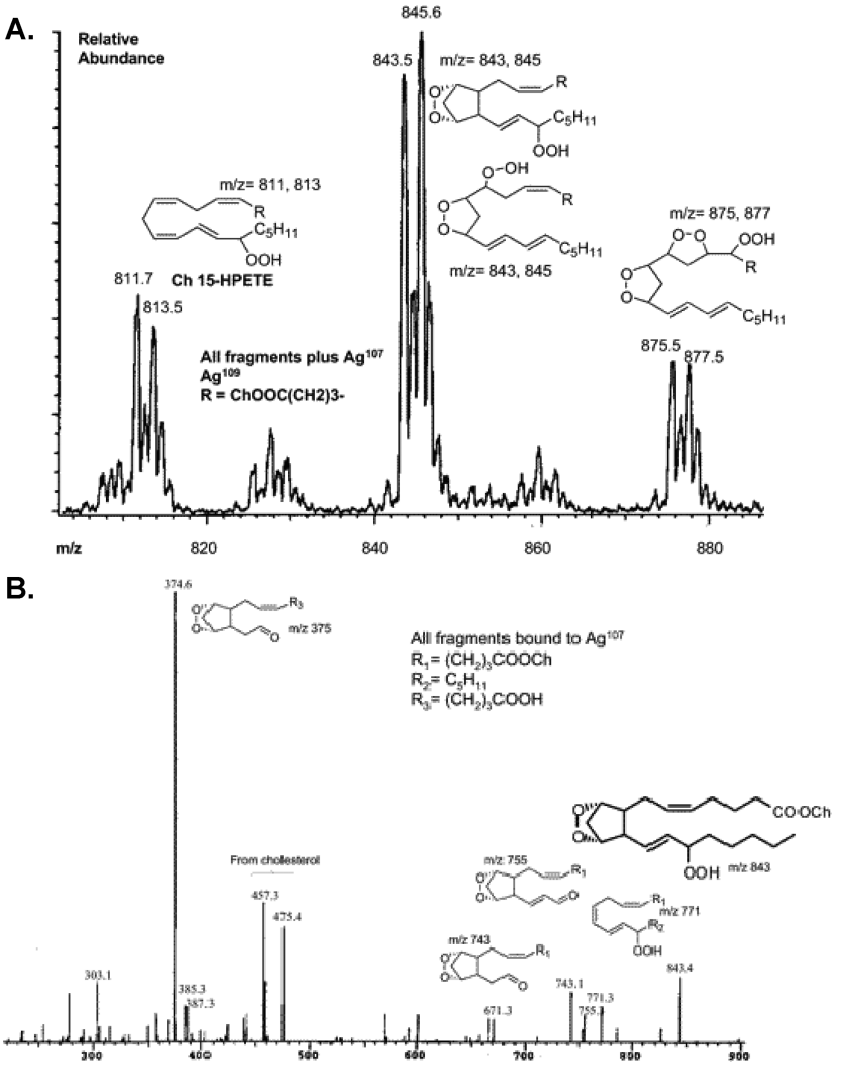


**Figure 8.** Identification of bicyclic endoperoxides from an oxidation mixture of the cholesterol ester of 15-HPETE by $Ag^+$-CIS−MS. (A) Parent ions of oxidation mixture by direct liquid infusion. (B) CID of $m/z$ 843.

$Ag^+$ CIS−MS has also been utilized to characterize lipophilic compounds including tocopherols and carotenoids after normal phase separation and postcolumn addition of silver ion. This method has been explored to study the lipid peroxidation products by coupling the MS with normal phase HPLC.[219,220]

In addition to the bicyclic endoperoxides, as noted earlier in this Review, a host of oxidation products can be formed from the oxidation of polyunsaturated lipids. Cholesterol arachidonate, for example, is expected to give products that incorporate multiple oxygen atoms upon peroxidation, and indeed the results from direct infusion MS of the silver ion solution of the oxidation mixture from this lipid show multiple products with the silver ion characteristic doublet. Thus, doublets at $m/z$ 843.5 and 845.6 are consistent with structures of the bicyclic endoperoxides and monocyclic peroxides, whereas the ions at $m/z$ 875.5 and 877.6 are signatures of serial cyclic peroxides. The structure of the bicyclic endoperoxides is supported by the fragmentation observed in collision induced dissociation (CID); see Figure 8B. When the oxidation mixture is subject to normal phase separation and post column addition of silver ion solution of $AgBF_4$ in isopropanol, CID was carried out for the ion of $m/z$ 843.5, and the characteristic fragment ions observed are consistent with the bicyclic endoperoxide structure.

As shown in Figure 8B, the characteristic fragments at $m/z$ 375 and 743 are derived from Hock cleavage of the hydroperoxides at C-15. Loss of malondialdehyder from the bicyclic endoperoxides generates fragments with $m/z$ 771 and 303, indicating the presence of bicyclic endoperoxide moiety in the molecules. Moreover, the $Ag^+$-CIS−MS has been successfully applied to identify other lipid oxidation products derived from EPA and DHA.[104] A novel series of oxidation products term dioxolane-IsoPs have also been identified from the oxidation of 12- and 8-HPETE by the use of the silver ion technique.[103]

### 4.3. LC−ESI−MS

ESI−MS has been widely used to study lipid oxidation products due to its sensitivity and specificity. ESI−MS can be easily coupled to reverse phase LC separation, and this technique has been employed to analyze oxidized phospholipids, neutral lipids such as triglycerides and cholesterol esters, and oxidation products from free fatty acids. Tandem MS/MS techniques


dx.doi.org/10.1021/cr200084z |*Chem. Rev.* 2011, 111, 5944–5972

enable the characterization of oxidation product when CID experiment was carried out in a triple quadruple, iontrap, or TOF mass analyzer. Quantification of specific oxidation products is normally carried out in a triple quadrupole instrument using selected reaction monitoring (SRM).[221] This technique significantly improves the specificity by monitoring the transition of parent molecular ion to a characteristic fragment in different quadrupoles.

Taking the $F_2$-IsoPs as example, four series of regioisomers can be formed from the oxidation of arachdionic acid, and each series has characteristic fragments in the CID spectrum. Thus, the SRM experiment has been employed to gain information of regisoisomers as well as diastereomers.[222]

The intact oxidation products on phospholipids have also been studied using the LC−ESI−MS technique. To characterize these phospholipid oxidation products, iontrap MS based on the $MS^n$ capability was generally employed.[223] The oxidation products from PE, PS, and PI can be readily detected in the negative ion mode, while PCs give a prominent response in the positive ion mode. In the negative ion mode, $MS^2$ generates the carboxylate ion of the fatty acids on the *sn*-1 and *sn*-2 glyceryl position. Similarly, quantification of the different oxidation products has been accomplished from different biological sources using the SRM techniques.

### 4.4. Electron Capture Atmospheric Pressure Chemical Ionization Mass Spectrometry (EC−APCI−MS)

APCI−MS is based on ionization by ion−molecule or electron capture reactions that are carried out in an ion source operating at atmospheric pressure ($10^5$ Pa). While ESI is generally used to ionize polar molecules, APCI ionization operates well for nonpolar analytes. Although the use of APCI is not as widespread as ESI, APCI−MS has proven to be a very valuable technique for analysis of lipids and other biological compounds, and it offers the possibility of interfacing with HPLC, which makes it an ideal tool for use in solving some difficult analytical problems. APCI−MS can also be operated in the electron capture negative ionization mode. This combination provides an increase in sensitivity of 2 orders of magnitude when compared to the conventional APCI methodology. The extraordinary sensitivity of EC−APCI−MS for a strong electrophore basically derives from the high efficiency of capture of a thermal or near thermal electron and formation of a dominant anion product. Pentafluorobenzylation is commonly used to derivatize biomolecules because it readily replaces active hydrogen, such as phenol, carboxylic acid, and heterocyclic NH, leading to a product that typically undergoes facile dissociative EC to form an analyte characteristic anion.[224] This strategy has also used to analyze PFB esters without TMS derivatization. EC−APCI−MS has been applied to study $F_2$-IsoPs when coupled with normal phase separation.

### 4.5. Analysis of Sterols and Oxysterols

Sterols and oxysterols were historically analyzed by GC−MS after trimethylsilyl derivatization. With the development and advance of MS techniques, HPLC−MS analysis is gaining in use for the analysis of oxysterols. Because of the difficulty in ionization of sterols, the mild ionization technique, ESI, can only be used when the sterols are derivatized. In contrast, APCI can be utilized as the ionization source for underivatized oxysterols.

The C-3 hydroxyl group of sterols has been derivatized to easily ionizable dansyl,[225] picolinyl,[226,227] glycinyl,[228] ferrocenecarbamate,[229] and sulfate esters[230] as well as the *N*-methylpyridinyl ether.[231] Indirect derivatization such as oxidation of the C-3-hydroxyl to ketone by cholesterol oxidase followed by formation of hydrazone has also been employed.[232] Detailed studies of these derivatization and analysis methods have been reviewed thoroughly by Griffiths and co-worker.[233] These derivatization methods, although somewhat time-consuming and perhaps unsuitable for unstable sterols such as hydroperoxides and endoperoxides, nevertheless provide high sensitivity for stable sterols when coupled with HPLC−ESI−MS analysis. For example, charged hydrazones of 3-oxo steroids could be detected at subpicorgram levels,[232] and picolinyl ester of oxysterols can be detected in the range of 2−10 fg.[226,227]

APCI can be used to monitor oxysterols directly, giving molecular ions and loss of one or multiple water ions. Through selective reaction monitoring (SRM), monitoring the loss-of-water transition in the mass spectrometry, the limit of detection by the use of this method can approach sub 100 pg.[226,234] The APCI method has the advantage of little manipulation of sample, shortened sample processing time, and monitoring multiple classes of oxysterols conveniently. Both normal phase and reverse phase HPLC can be coupled to the APCI source, which provides more options to achieve optimum separation of oxysterols. The APCI method proved to be especially useful in analyzing 7-DHC-derived oxysterols, which contain 1, 2, 3, or 4 additional oxygen atoms relative to the parent 7-DHC.[59] The oxysterols with different numbers of oxygen atoms can be differentially monitored with SRM.[59]

In summary, detection and quantification of lipid oxidation products in vitro and in vivo brings a unique set of challenges. A number of MS-based techniques have been developed for the analysis of various oxidation products. These complementary methods offer options for different analytes. GC−MS generally has the highest sensitivity but requires extensive prepurification and derivatizations, while APCI−MS offers some unique advantages for detection and chromatographic separations.

## 5. SUMMARY

Lipid present in membranes and aggregates in biological tissues and fluids is a complex mix of classes and molecular species. Phospholipids, triglycerides, and cholesterol esters are comprised of multiple fatty acid esters that result in a mixture of molecular species numbering well over 1000. Exposure of this complex lipid mixture to sources of free radicals and molecular oxygen results in a chain reaction, lipid peroxidation that gives rise to a myriad of products.

The initiation of free radical reactions and their inhibition in biology has been the focus of intensive research activity over the past half century, and an understanding of the important processes that begin and end radical chain reactions has emerged and continues to develop. On the other hand, the chemistry associated with the lipid targets of free radicals in biology has remained, for the most part, in a black box. One reason for the difficulty in the investigation of lipid peroxidation has been the complexity of the product profile that can be formed from a single molecular species and the limitations of the tools that have been available for analysis of the complex product mixtures. Cholesterol arachidonate, for example, is one of the lipids found in lipoproteins, and literally hundreds of products are formed in the free radical chain oxidation of this single molecular species. Oleate, linoleate,

eicosapentaenoate, and docosahexaenoate cholesterol esters are also present in lipoproteins, and multiple products are formed from these molecular species. Furthermore, cholesterol esters are only one of the lipid classes that will be a target for radical chain processes; the analytical problem is overwhelming.

The development of powerful mass spectrometry tools, used in conjunction with HPLC separations, has, however, allowed serious efforts to unravel complex product profiles and to address fundamental chemical mechanistic questions about peroxidation. What emerges from these studies is an understanding of product profiles and mechanism based upon established transformations that are familiar to free radical chemists. Reversible oxygen addition to carbon free radicals, H-atom transfer to propagating peroxyls, peroxyl radical addition and cyclization, and intramolecular carbon radical attack on the peroxide bond are well-known transformations that account for most of the primary products formed from natural lipids.

Many of the primary products formed in free radical chain oxidation of lipids are, however, unstable peroxidic compounds that are difficult to isolate and identify. Yet it is these unstable products that are the first-formed products of an assault on an organism by a radical initiation event, and it is this product profile that must be processed and metabolized by the organism. To illustrate the point, peroxidation of a cholesterol arachidonate molecule gives rise to hundreds of primary products that are comprised of cyclic peroxides, bicyclic endoperoxides, and hydroperoxides, among other products. These first-formed products, which may well have potent biological activities, are unstable and are processed or decompose to give downstream compounds. For example, hydroxyl-substituted lipid esters, malondialdehyde, $F_2$-IsoPs, and a variety of electrophilic compounds such as 4-HNE are formed from decomposition of the primary peroxide products of cholesterol arachidonate.

The isolation of byproducts of lipid peroxidation such as the isoprostanes, the gold standard for oxidative stress, and the cytotoxic 4-HNE is evidence for the existence of the process, but much of the biology of oxidative stress and lipid peroxidation lies under the surface because the primary product set, the peroxides, hydroperoxides, and epoxides that are undoubtedly generated by the process, has not been isolated and their biology characterized. The isoprostanes are reasonably resistant to metabolism and processing and they are useful biomarkers for that reason, but they are the surviving detritus of peroxidation that represents only a fraction of the underlying chemistry and biology that attends oxidative stress processes. 4-HNE is a potent cytotoxin, but it is but one of the many electrophiles that are likely to form in a cell undergoing oxidative assault. It seems likely that many other potent compounds having unique biological activities are formed, and developing methods for associating biological activity to the hundreds of compounds formed in peroxidation of a lipid mixture is one of the many challenges of research in the field going forward. The new tools for analysis that have been made available in the past decade permit some of these problems to be approached, but additional tools are needed to continue the exploration of these important questions.

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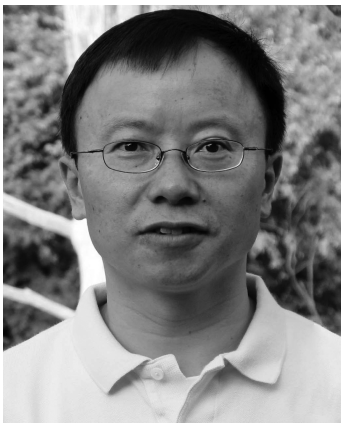
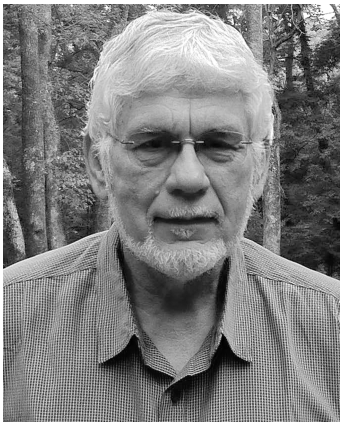
## BIOGRAPHIES



Huiyong Yin graduated from Tongji University, Shanghai, China, with a B.S. in Chemistry in 1992. In 1995, he received his M.S. degree from the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences (CAS). After receiving his Ph.D. with Dr. Ned Porter at Vanderbilt University in 2002, he joined Dr. Jason Morrow's laboratory in the Division of Clinical Pharmacology at Vanderbilt School of Medicine as a research instructor and was promoted to research assistant professor in 2005 and research associate professor in 2009 in the Departments of Medicine, Pharmacology, and Chemistry. He was the director of Drug Metabolism and Pharmacokinetics (DMPK) analysis for the Vanderbilt Program of Drug Discovery from 2007 to 2009. In 2011, he moved to the Shanghai Institute of Nutritional Science, Shanghai Institute for Biological Sciences (SIBS), CAS, where he is a professor and the Director of the Mass Spectrometry Research Center for Nutrition and Metabolism. His main research interest focuses on free radical mechanisms of lipid oxidation and its role in human health and disease, and the application of mass spectrometry techniques including lipidomics, metabolomics, and proteomics to studies of nutrition and metabolism. He is also interested in the organic synthesis of bioactive lipids, DMPK, and preclinical drug discovery.

Dr. Libin Xu received his B.Sc. in Chemistry from Nankai University, China, in 2002. He started his research on reactions involving radicals and radical ions in the same year under the guidance of Dr. Martin Newcomb at the University of Illinois at Chicago, where he was awarded a Ph.D. in Physical Organic Chemistry in 2007. He subsequently joined the laboratory of Dr. Ned Porter at the department of chemistry at Vanderbilt University and is currently a research assistant professor in the

department. Dr. Xu's research interests involve the mechanism of free radical reaction, lipid peroxidation (especially oxidation of sterols), the mechanism of formation and biological activities of oxysterols in vitro and in vivo, and Smith−Lemli−Opitz syndrome. Among the awards he has received are the Young Investigator Award from the Society for Free Radical Biology and Medicine (2010) and the Joseph M. and Eula C. Lawrence Travel Grant Award from the ARVO Foundation for Eye Research/Retina Research Foundation (2011).



Ned Porter graduated from Princeton in 1965 with a B.S. in Chemical Engineering and accepted a position as Assistant Professor at Duke University in 1969, after receiving his Ph.D. with Paul D. Bartlett at Harvard. In 1998, he moved to Vanderbilt University where he is Stevenson Professor of Chemistry. His research interests have centered on the mechanisms of free radical reactions. A continuous research theme has been on the interplay between free radical chemistry, lipids, and oxidative stress in biology.

## ABBREVIATIONS

AAPH 2,2′-azobis(amidinopropane) dihydrochloride
AMVN 2,2′-azobis(2,4-dimethylvaleronitril)
APCI atmospheric pressure chemical ionization
APPI atmospheric pressure photoionization
BDE bond dissociation enthalpy
BHT butylated hydroxytoluene
CD36 Cluster of Differentiation 36
CDPX2 X-linked dominant chondrodysplasia punctata
CID collision-induced dissociation
CIS coordination ionspray
COX cyclooxygenase
CTX cerebrotendinous xanthomatosis
CYP3A4 cytrochrome P450 3A4
DHA docoshexaenoic acid
7-DHC 7-dehydrocholesterol
8-DHC 8-dehydrocholesterol
DHCAO 3$\beta$,5$\alpha$-dihydroxy-cholestan-6-one
DHCEO 3$\beta$,5$\alpha$-dihydroxycholest-7-en-6-one
DFT density functional theory
DTBN di-*tert*-butylhyponitrite
EC electron capture
EPA eicosapentadienoic acid
ESI electrospray ionization
ESR electron spin resonance
ETC electron transport chain
FAB fast atom bombardment
FOX ferrous oxidation of xylenol
GC gas chromatography
HDdiA-PC 9-hydroxy-10-dodecenedioic acid esters of 2-lysoPC
HOdiA-PC 5-hydroxy-8-oxo-6-octenedioic acid esters of 2-lysoPC
HODA-PC 9-hydroxy-12-oxo-10-dodecenoic acid esters of 2-lysoPC
HOOA-PC 5-hydroxy-8-oxo-6-octenoic acid esters of 2-lysoPC
HETE hydroxyeicosatetraenoate
4-HNE 4-hydroxynonenal
HODE hydroxyoctadecadienoic acid
HPLC high performance liquid chromatography
HPETE hydroperoxyl-eicosatetraenoate
HPODE hydroperoxyoctadecadienoic acid
IsoP isoprostane
KODA-PC 9-keto-12-oxo-10-dodecenoic acid esters of 2-lysoPC
KDdiA-PC 9-keto-10-dodecendioic acid esters of 2-lysoPC
KOdiA-PC 5-keto-6-octendioic acid esters of 2-lysoPC
KOOA-PC 5-keto-8-oxo-6-octenoic acid esters of 2-lysoPC
KOH potassium hydroxide
LC liquid chromatography
LDL low density lipoprotein
MALDI matrix-assisted laser desorption/ionization
MeOAMVN 2,2′-azobis(4-methoxy-2-dimethylvaleronitril)
MDA malondialdehyde
MS mass spectrometry
NADPH nicotinamide adenine dinucleotide phosphate
PAPC 1-palmitoyl-2-arachidonyl-phosphotidylcholine
PC phosphotidylcholine
PE phosphotidylethanolamine
PFB pentafluorobenzyl
$PGF_{2\alpha}$ prostaglandin $F_{2\alpha}$
PI phosphotidylinosotol
PS phosphotidylserine
PUFA polyunsaturated fatty acid
ROS reactive oxygen species
$S_Hi$ intramolecular homolytic substitution
SLOS Smith−Lemli−Opitz syndrome
SRM selected reaction monitoring
TBARS thiobarbituric acid reactive substance
TMS trimethylsilyl
TOF time-of-flight
UV ultraviolet

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